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Correlation of micronucleus and apoptosis assays with reproductive cell death

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Abstract. The relationship between ionizing radiation-induced cell killing and DNA damage measured by the micronucleus and apoptosis assays was determined in three established cell lines (L929, HL-60, and Chang). Irradiation experiments revealed a dose-dependent increase of micronucleated cells until a certain dose was reached. Above this dose no further increase of the micronucleus frequency was observed, but in HL-60 and Chang cells additional DNA fragmentation was detected by morphological criteria, characteristic of apoptosis. This change was detected at different doses for the three cell lines examined, suggesting the existence of a cell-type-dependent upper limit for the employment of the micronucleus assay. However, the sum of both kinds of cellular DNA damage (e.g. micronucleation and morphological-like apoptosis) led to a significant cell-type-independent correlation with cell survival, even above the dose where micronuclei levels saturated. Therefore, a total cell damage assay, involving the inclusion of micronuclei and morphological-like apoptotic events, should be considered when evaluating the use of a predictor assay for ionizing radiation-induced cell killing, especially in conditions when apoptosis (-like) processes may occur.

1. Introduction

The micronucleus (MNC) assay has been used in the fields of biological dosimetry and as a biological indicator for radiosensitivity for as long as three decades (e.g. Fliedner *et al.* 1964, Schmid 1975, 1976). In parallel, MNC dose–response curves for human cells were constructed (Countryman and Heddle 1976, Krepinsky and Heddle 1983). The survival/dose–response relationship with MNC differs from report to report, depending on the cell lines observed. In human lymphocytes the relationship confirmed to the power law expression (Countryman and Heddle 1976), while in the bone marrow of mouse it was linear (Jenssen and Ramel 1976, 1978, Cole *et al.* 1981, Jagetia 1990). It is suggested that these differences are partly due to cell cycle perturbations induced by clastogens/aneugens. In order to overcome the cell kinetic problem, the cytochalasin B (CB) method (Fenech and Morley 1985) and the bromodeoxyuridine (BrdU) method (Pincu *et al.* 1984) were introduced. The CB-Block method is widely used since it enables those cells that have attempted mitosis to be distinguished from those that have not, so that the latter may be excluded from analysis.

There is still a controversy regarding the potency of this modified MNC assay as an indicator of radiosensitivity (e.g. van Beuningen *et al.* 1981, Streffer *et al.* 1982, Bush and McMillan 1993), indicating that unknown factors may influence the assay. Apoptosis (controlled cell death) has been known since 1972 (Kerr *et al.* 1972). It is hypothesized that, through the release of a still not fully defined endonuclease, DNA fragmentation occurs (for detailed discussion, see the review by Haake and Polakowska 1993). The relevance of apoptosis to cell survival, e.g. after irradiation of cells and tissue of different origins, is well described (for a review, see Sen 1992). However, the contribution of apoptosis to the survival/dose–response-relationship of radiation-induced MNC formation is unknown.

In this study, we compared for the first time the radiation-induced yield of both MNC and apoptosis with survival (clongenicity was used as an indicator for individual radiosensitivity) of three cell lines from various tissue types (i.e. fibrosarcoma, epithelial-like liver, myeloid leukaemia). The sum of micronucleated cells and apoptotic events, total cell damage (TCD) assay, was tested to determine its prediction ability for radiation effectiveness over a dose range that resulted in about two decades of cell killing.

2. Materials and methods

2.1. *Cells and cell culture*

Mouse fibrosarcoma (L929) and human epithelial-like liver cells (Chang) (Flow Laboratories, Germany) were each grown as a monolayer in Earle's modification

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of Eagle's minimum essential medium (MEM) supplemented with 20% heat-inactivated foetal calf serum and subcultured twice per week. Myeloid leukaemia (HL-60) cells (a gift from Professor Grosse-Wilde, University Essen) were grown in RPMI 1640 medium supplemented with 20% heat-inactivated foetal calf serum and subcultured twice per week. Culture conditions were 37°C in a humidified atmosphere buffered by 5% CO_2 in air and hydrogen carbonate, pH 7·4. All chemicals for media were purchased from Boehringer (Germany).

2.2. *Radiation conditions*

Twenty-four hours after passaging, exponentially growing cell cultures were irradiated at room temperature with a single dose of 240 kV X-rays (Isovolt 320/10; Seifert, Germany) filtered with 3 mm Be. Absorbed dose was measured with a Duplex dosimeter (PTW, Germany). The dose-rate was about 1 Gy/min at 13 mA. L929 and Chang cells were irradiated with 2, 4 and 6 Gy, and HL-60 cells with 0·1, 0·3, 0·5, 1·0, 1·5, 2·0 and 2·5 Gy (doses were selected to obtain comparable survival levels; Figure 1).

2.3. *Cell survival*

Cell survival was measured by standard clonogenic cell survival assay. The survival/dose–response curves were fitted by a linear–quadratic model for L929 and Chang cells, but a linear model for the HL-60 cells. Radiation sensitivity parameters (D_0 and D_q) were determined from a tangent line fitted to the terminal portion of the survival/dose–response data (Hall 1973).

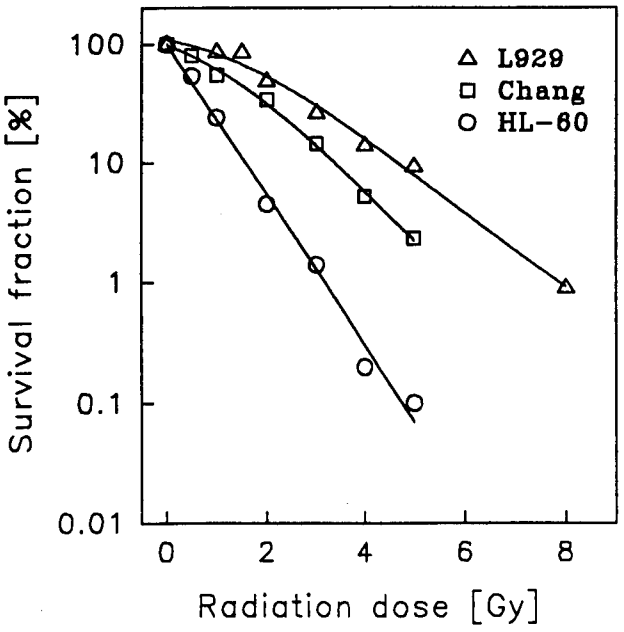


Figure 1. Cell survival radiosensitivity (240 kV X-ray). Symbols reflect the mean of at least three experiments. Typical SEM was < 2%.

2.4. *Micronucleus assay*

Cells were trypsinized when needed (L929 and Chang cells) after aspiration of the cell-free medium at certain time points after irradiation as indicated above. Trypsin was inactivated by adding the same volume of medium, centrifuged and rinsed twice in PBS (phosphate-buffered saline). Cells were gently cytospined (< 50*g*, 5 min) afterwards, air dried (10 min), fixed in methanol (room temperature, 10 min), incubated in DAP solution (4′,6-diamidino-2-phenylindole; Serva, Germany) for 5 min, washed in distilled water (5 min), air dried again (10 min), and stored at 4°C. The slides were scored for MNC (magnification × 400) with the aid of an epifluorescence microscope (Diaplan 20, Leica, Germany) equipped with an UV filter (excitation at 270–380 nm, emission at 410–580 nm). The criteria for scoring MNC agreed with, e.g. Hennig *et al.* (1988). They included sharply bordered nuclei and a spatial separation between the MNC and the cell nucleus. The MNC had to be ≤ 20% of the nucleus diameter, and the MNC's fluorescence had to have the same colour and intensity as the nucleus. MNC that were difficult to discriminate from protrusions were not considered and neither were radiation-induced binucleated cells containing more than one MNC.

We did not perform the CB method for reasons described in §4. Instead, the formation of MNC was determined at different time points (time interval was 2–3 h) from 0 to 50 h after irradiation. The number of MNC formed per 100 cells (MNC%) as well as number of micronucleated cells per 100 cells (MC%) were determined at different time points as described by Almassy *et al.* (1987). Each experiment was performed three times. For each data point, 1500 cells were scored.

2.5. *Apoptosis assay*

Apoptotic cells were counted microscopically, according to published criteria (Falkvoll 1990, Stephens *et al.* 1991, Meyn *et al.* 1993). The frequency was expressed as the number of apoptotic cells per 100 cells. Per data point, 1500 cells were counted. Additionally, apoptosis was evaluated by gel electrophoresis as described by Ramakrishnan *et al.* (1993).

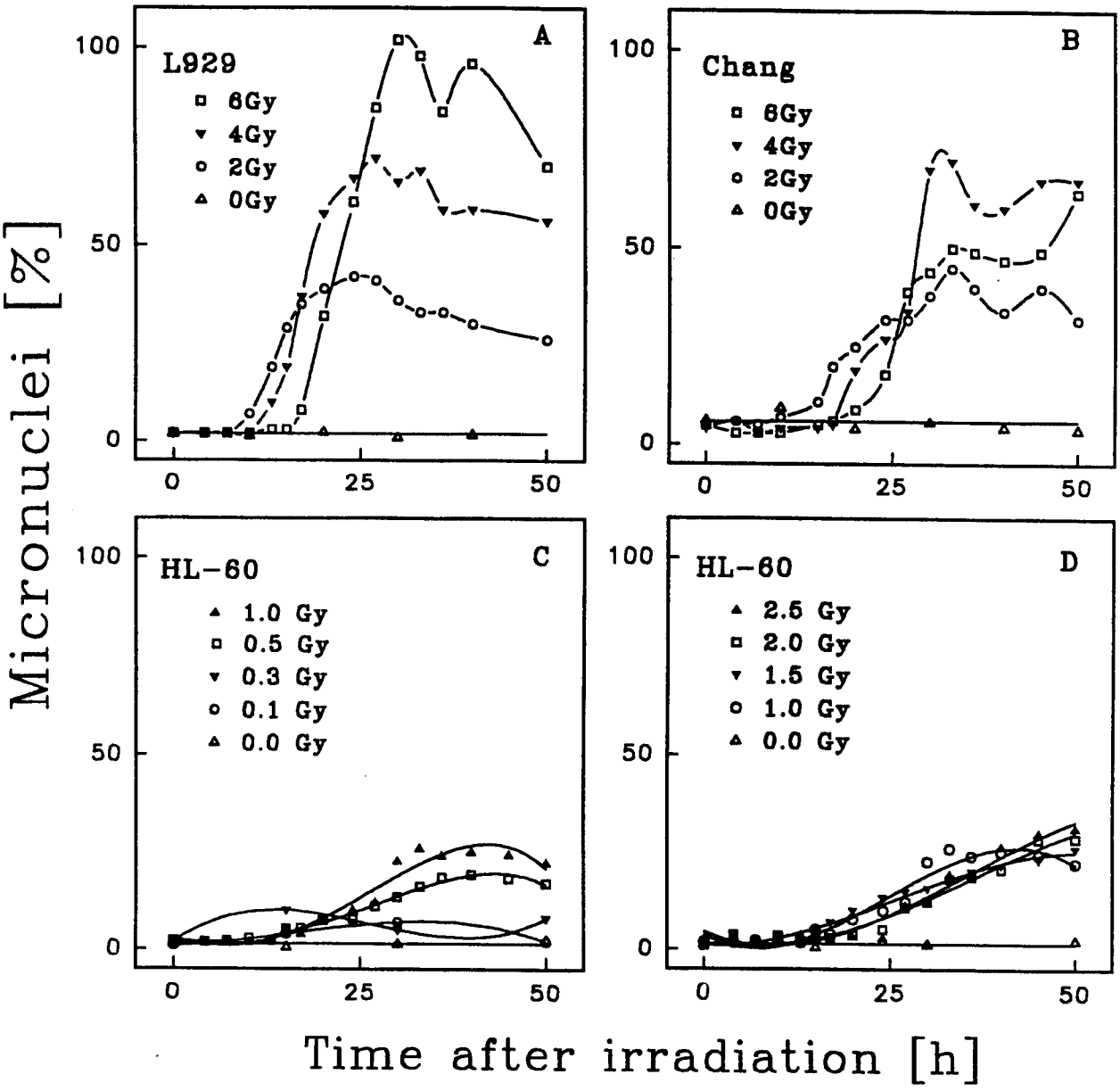


Figure 2. Comparison of micronucleus (MNC) frequency per 100 cells (MNC%) versus time after irradiation at different doses for L929 cells (a), Chang cells (b), and HL-60 cells (c and d). Typically, SEM was $< 3{\cdot}9\%$ in L929 and Chang cells and $< 1{\cdot}5\%$ in HL-60 cells. The curves in a and b were obtained by a spline fit connecting sequential data points. The curves in c and d were determined using a polyomial fit through the data points. A process of saturation in the formation of MNC% was detected in Chang ($> 4{\cdot}0$ Gy) and in HL-60 ($> 1{\cdot}0$ Gy) cells, but not in L929 cells.

2.6. *Total cell damage*

Total cell damage (TCD) represents the sum of the number of cells exhibiting micronucleation and apoptotic events per 100 cells (TCD%).

2.7. *Statistics*

Cell survival and cell damage points were based on at least three experiments. The means, standard error, significance levels (*t*-test) as well as the non-weighted-by-variance fit of curves were calculated with the aid of statistical software (Sigma Plot, 5·0, Jandel, Germany).

3. Results

3.1. *Survival assay*

The X-ray survival radiosensitivity for the L929 cell line (Figure 1) was characterized by a shoulder with

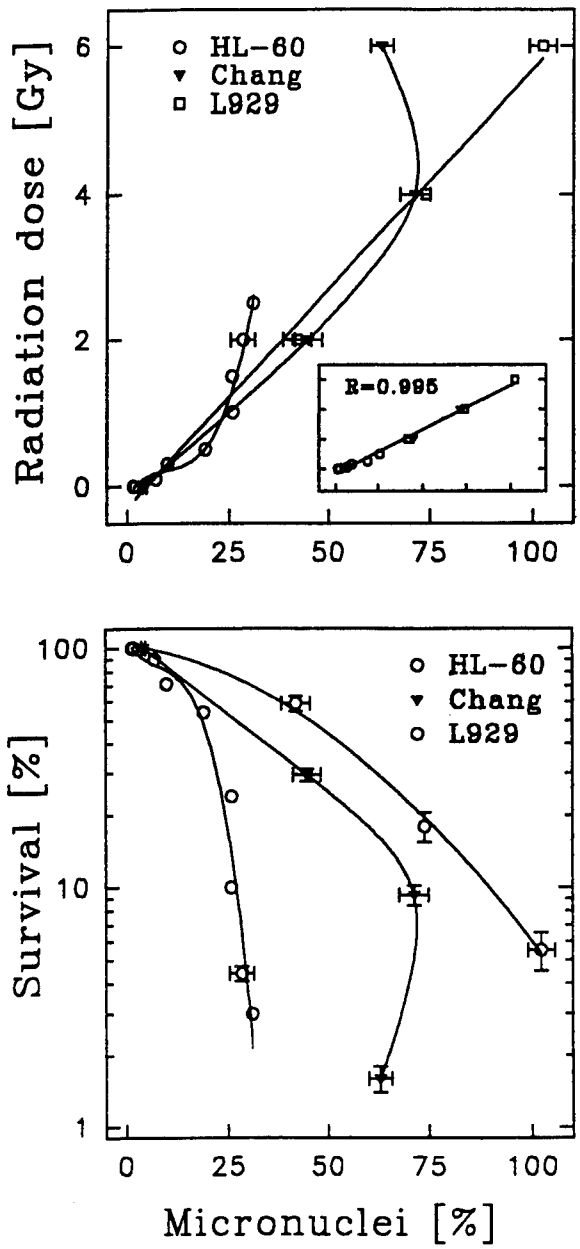


Figure 3. Correlation of the maximum MNC-frequency per 100 cells (MNC%) with radiation dose and survival. Maximum MNC%'s, were obtained from the time-course measurements from Figure 2. The upper and lower graphs represent individual polynomial curve fits for HL-60 and L929 (with $p < 0{\cdot}05$). Chang data were connected through a spline curve. The inset displays a linear curve fit through all data points of the cell lines characterized by the absence or a low number of detected apoptotic cells ($f(x) = 0{\cdot}056 - 0{\cdot}344x$; $R = 0{\cdot}995$; $p < 0{\cdot}05$). SEM of MC% data (x-bar) and SEM of survival (y-bars, lower graph) are visible when greater than the symbols. Both graphs reveal a process of saturation in the formation of MNC% in Chang (about 75%) and HL-60 (about 25%) cells, but not in L929 cells.

$D_q = 1{\cdot}47 + / - 0{\cdot}03$ Gy. The Chang cell line revealed a smaller shoulder ($D_q = 0{\cdot}97 + / - 0{\cdot}06$ Gy). To the contrary, no shoulder was observed in the HL-60 cell line. The latter reflected also the highest radiosensitivity ($D_0 = 0{\cdot}67 + / - 0{\cdot}09$ Gy) of the three cell lines examined, followed by the Chang ($D_0 = 1{\cdot}1 + / - 0{\cdot}06$ Gy) and the L929 cell lines ($D_0 = 1{\cdot}2 + / - 0{\cdot}15$ Gy). Thus, the cell lines were different with regard to their radiosensitivity for survival.

3.2. *MNC% data set*

3.2.1. *MNC% versus time after irradiation.* The MNC% peak increased dose dependently in the L929 cells from 42 to 74 and 102% at 2, 4, and 6 Gy respectively (Figure 2a). Chang cells revealed a MNC% peak of 44% at 2 Gy and 71% at 4 Gy. The MNC% frequency after irradiation with 6 Gy was measured up to 80 h post-exposure. The highest MNC% yield was 63% detected at 50 h post-exposure (data > 50 h post-exposure are not shown for better comparison; Figure 2b). The MNC% peak of HL-60 cells increased until a dose of 1·0 Gy was reached (26%; Figure 2c) After higher doses, the MNC% frequency remained comparable with the 1·0-Gy values at doses up to 2·5 Gy (26–31%, Figure 2d). Additional experiments, performed during the first 72 h post-exposure in higher dose HL-60 cells (1·5–2·5 Gy dose range), revealed no further increase of MNC%.

Hence dose-dependent increase of the MNC% frequencies seemed to be restricted to 1·0 Gy in the radiosensitive HL-60 cells, to 4 Gy in the less radiosensitive Chang cells and to > 6 Gy in the most radioresistant L929 cells. Flow cytometry measurements of proliferation kinetics (Gilbertz *et al.* 1993, Abend unpublished data), the mitotic index and the counts of the cell number after irradiation revealed the release of all irradiated cells from the G_2 block at 30 h and earlier post-exposure. In detail, the mitotic index (MI, defined as number of metaphases counted per 100 cells) decreased from controls of $1{\cdot}1 + / - 0{\cdot}2\%$ in L929, $1{\cdot}6 + / - 0{\cdot}2\%$ in Chang cells and $1{\cdot}1 + / - 0{\cdot}1\%$ in HL-60 cells at 0 h after irradiation to values near 0% during the following 10–17 h (depending on the radiation dose). Thereafter, a sudden increase to around 2% was measured in irradiated (2, 4 and 6 Gy) L929 cells, Chang cells and HL-60 cells irradiated with < 1 Gy in contrast with higher irradiated HL-60 cells (with an *MI* of about 1%). The MI decreased afterwards, but, in general, remained above control values up to 40–50 h after irradiation. L929 cell growth curves revealed a cell number doubling time of 22, 23·3, 24·7 and 27·5 h after irradiation with 0, 2, 4 and 6 Gy respectively. The Chang cell number doubling times were 21, 22, 27 and 30 h after irradiation with 0, 2, 4 and 6 Gy respectively. The cell number doubling times for HL-60 cells were

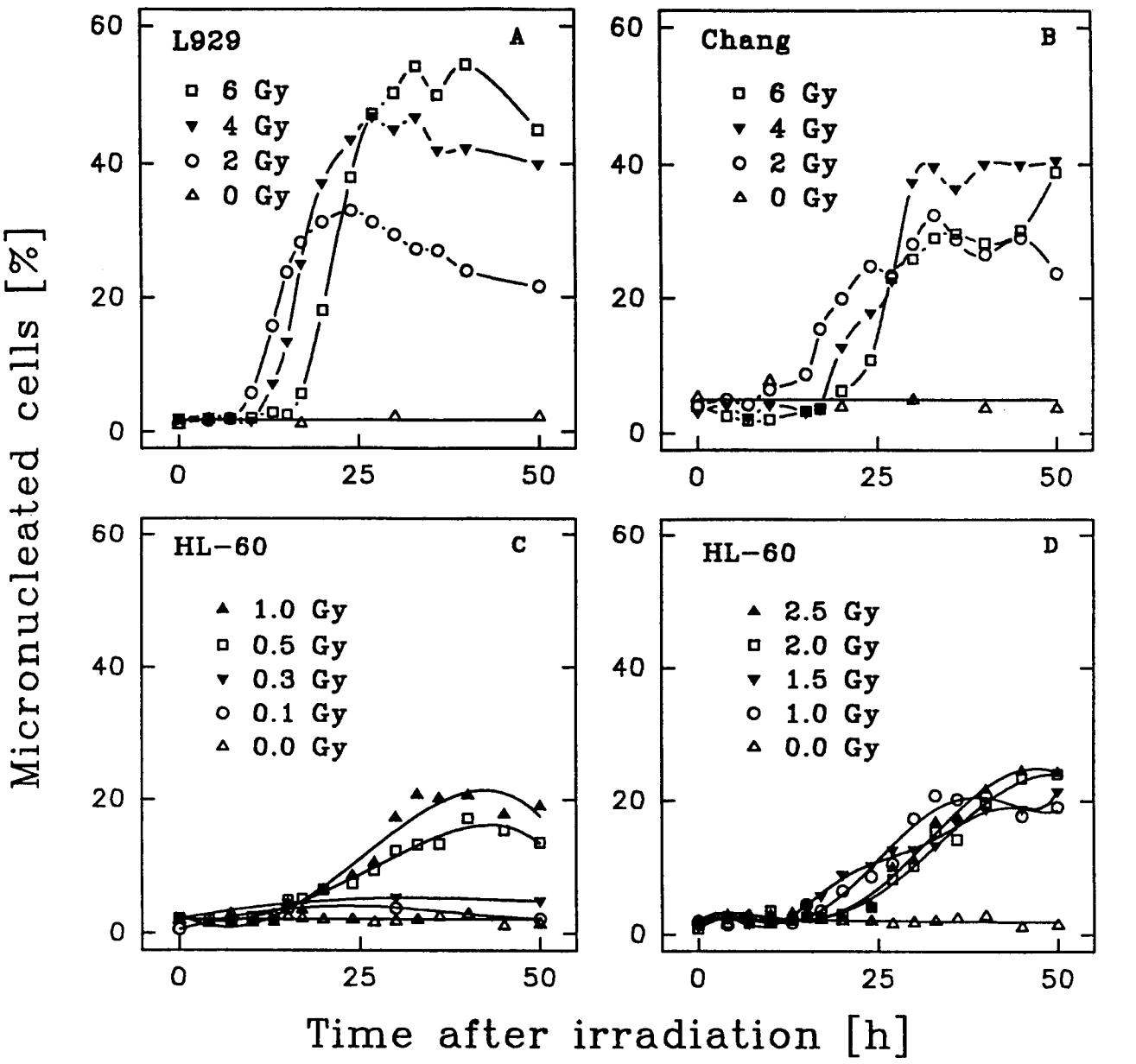


Figure 4. Comparison of the number of micronucleated cells per 100 cells (MC%) versus time after irradiation at different doses for L929 cells (a), Chang cells (b), and HL-60 cells (c and d). Typical SEM was $< 1{\cdot}5\%$. The curves in a and b were obtained by a spline fit connecting sequential data points. The curves in c and d were determined using a polynomial fit through the data points. A process of saturation in the formation of MC% was detected in Chang ($> 4{\cdot}0$ Gy) and in HL-60 ($> 1{\cdot}0$ Gy) cells, but not in L929 cells.

24, 29, 36, 41, 88 and 136 h, measured at radiation doses of 0, 0·5, 1·0, 1·5, 2·0, and 2·5 Gy respectively. Utilizing a BrdU/DNA approach, it was shown that a considerable amount of HL-60 cells left the cell cycle, which explains the high yield of the cell number doubling time after irradiation with > 1 Gy (data not shown).

3.2.2. *MNC% correlated with radiation dose and survival.* The relationship between the maximum yield of MNC%, derived from the time-course measurements (Figure 2), and radiation dose or survival demonstrate a distinct cell-line dependence (the niveaus of significance of the individual curve fits of HL-60 and L929 MNC% data with survival and radiation dose were $p < 0{\cdot}05$, Chang data were connected by a spline curve). When omitting data points that fall within a dose range, where saturation of micronuclei formation was observed (6-Gy value of Chang cells and 1·5–2·5-Gy values of HL-60 cells), a significant linear fit of MNC% with radiation dose was obtained ($R = 0{\cdot}995$, $p < 0{\cdot}05$, $f(x) = 0{\cdot}056 - 0{\cdot}344x$; Figure 3, inset).

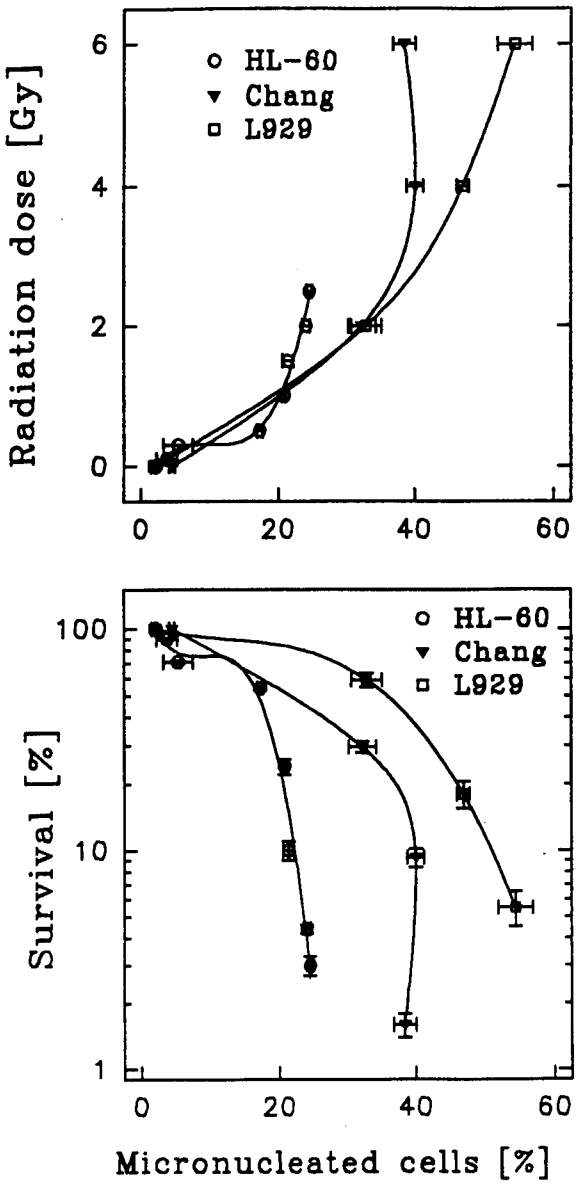


Figure 5. Correlation of the maximum number of micronucleated cells per 100 cells (MC%) with radiation dose and survival. Maximum MC%'s were obtained from the time-course measurements from Figure 4. The upper and lower graphs represent individual polynomial curve fits for HL-60 and L929 (with $p < 0{\cdot}05$). Chang data were connected through a spline curve. SEM of MC% data (*x*-bars) and SEM of survival (*y*-bars, lower graph) are visible when greater than the symbols. Both graphs reveal a process of saturation in the formation of MC% in HL-60 (about 20%) and Chang (about 37%) cells, but not in L929 cells.

3.3. *MC% data set*

3.3.1. *MC% versus time.* L929 cells revealed a MC% peak of 33, 47 and 54% at 2, 4 and 6 Gy, respectively (Figure 4a). The peaks observed in Chang cells were 33% at 2 Gy, 41% at 4 Gy, and 39% at 6 Gy. Though we measured the MC% of Chang cells up to 72 h after irradiation with 6 Gy, no further increase $> 34\%$ was detected. The highest yield of MC% of HL-60 cells were 4, 5, 17, 21, 24 and 25% measured at 0·1, 0·3, 0·5, 1·0, 1·5, 2·0 and 2·5 Gy respectively (Figure 4c, d). Additional experiments on HL-60 cells, performed during the first 72 h post-exposure, revealed no further increase of MC%. As shown for the MNC% data, a saturation in formation of MC% seemed to occur in Chang cells irradiated with 4 Gy and HL-60 cells irradiated with $> 1{\cdot}0$ Gy.

3.3.2. *MC% correlated with radiation dose and survival.* Each cell line revealed an individual correlation with the radiation dose (Figure 5, upper, the niveaus of significance of the individual curve fits of HL-60 and L929 MC% data with radiation dose were $p < 0{\cdot}05$. Chang data were connected by a spline curve). However, no significant correlation of all data points could be calculated even when we omitted data points that suggest a saturation in the formation of micronucleated cells.

The correlation of MC% with survival reflected a pattern similar to the MNC% data set (Figure 5, lower, the niveaus of significance of the individual curve fits of HL-60 and L929 MC% data with survival were $p < 0{\cdot}05$. Chang data were connected by a spline curve).

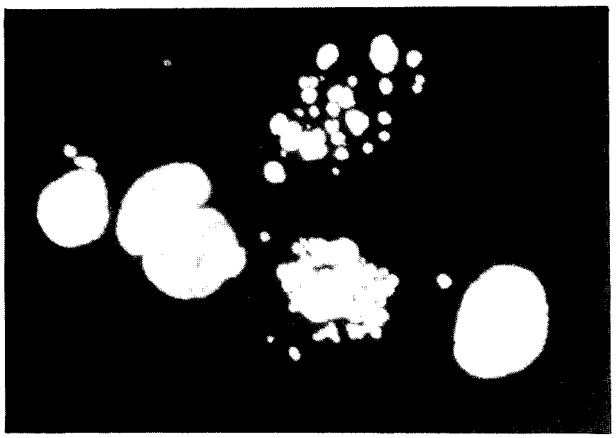
3.4. *Apoptosis and TCD data set*

3.4.1. *Detection of apoptotic cells.* Figure 6 represents the known features of DNA condensation and formation of apoptotic bodies in Chang and HL-60 cells. Even after irradiation with 20 Gy, it was not possible to detect apoptotic L929 cells morphologically and electrophoretically (Figure 7).

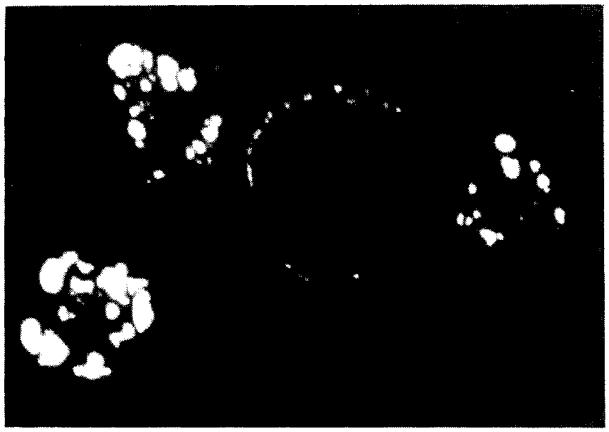
Though we were able to visualize the characteristic DNA ladder induced by apoptotic processes in HL-60 cells, no DNA ladder was observed in either Chang or L929 cells (Figure 7).

3.4.2. *Apoptosis versus time.* No increase of apoptotic Chang cells above the spontaneous level (0·3%) was detected up to 4 Gy (Figure 8a). However, irradiation with 6 Gy led to an increase of apoptotic cells up to 12%. Additional measurements performed up to 80 h after irradiation with 6 Gy revealed no further increase in apoptotic cells though Figure 8a suggested that the maximum yield may have not been reached at 50 h after irradiation (data not shown).

The percentage of apoptotic HL-60 cells rose dose-dependently from 13% at 0·5 Gy to 40% at 2·5 Gy (Figure 8b). The increase of apoptotic cells occurred at about 10–17 h post-exposure and was independent of the radiation dose applied.



(a)



(b)

Figure 6. Microscopic photograph of Chang and HL-60 cells stained with DAPI 30 h after irradiation (original magnification × 1000). (a) Upper part reflects an apoptotic Chang nucleus. A Chang cell with two micronuclei is shown below. (b) Normal-shaped HL-60 nucleus (Centre) surrounded by apoptotic bodies.

3.4.3. *TCD% versus time.* TCD% data (Figure 9) revealed a pattern similar to the MNC% and MC% measurements when examined versus time. However, TCD% data showed an increase through the whole dose range, even at 6 Gy in the Chang cells and > 1·0 Gy in HL-60 cells. In detail, the maximum TCD% data in Chang cells were 5, 33, 42 and 51% at radiation doses of 0, 2, 4, and 6 Gy respectively. The maximum TCD% induced in HL-60 cells by radiation doses of 0·0, 0·1, 0·3, 0·5, 1·0, 1·5, 2·0 and 2·5 Gy were 2, 7, 9, 28, 37, 43, 60 and 59% respectively.

3.4.4. *TCD% correlated with radiation dose and survival.* The parameters derived from a linear–quadratic fit of the relationship between radiation dose and maximal TCD% for Chang and L929 cell data were not significantly different ($p > 0{\cdot}05$)

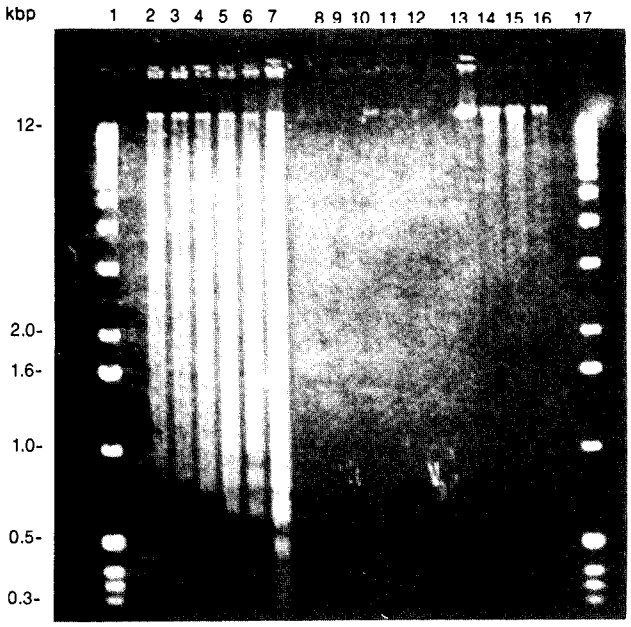


Figure 7. Agarose gel electrophoresis of DNA isolated from HL-60, L929 and Chang cells at 36 h after irradiation at different doses. Lanes 1 and 17, 1 kbp DNA standard (Gibco, BRL), supernatant DNA of HL-60 cells irradiated with: lanes 2, 0; 3, 0·5; 4, 1·0; 5, 1·5; 6, 2·0; and 7, 2·5 Gy; supernatant DNA of L929 cells irradiated with: 8, 0; 9, 2; 10, 4; 11, 6; and 12, 20 Gy; and supernatant DNA of Chang cells irradiated with: 13, 6; 14, 4; 15, 2; and 16, 0 Gy respectively.

in contrast with the results from the HL-60 cell data (Figure 10, upper, the niveaus of significance of the individual curve fits of HL-60, Chang and L929 TCD% data with radiation dose were $p < 0{\cdot}05$). Nevertheless, after plotting TDC% versus the survival of each cell line separately (Figure 10, lower, the niveaus of significance of the individual curve fits of HL-60, Chang and L929 TCD% data with survival were $p < 0{\cdot}05$), it became evident that all plots converged at one highly significant curve fit ($R = 0{\cdot}934$, $p < 0{\cdot}05$, $\log f(x) = 2{\cdot}0114 - 0{\cdot}0036x - 0{\cdot}000386x^2$, with '$\log f(x)$' which represents the survival rate in percent expressed as log to the base 10, and 'x', which represents TCD%) as shown in Figure 10 (inset).

Hence, the TCD% was the only parameter that (1) correlated with the radiosensitivity (survival), but was (2) independent of the three cell lines observed.

4. Discussion

There is controversy about the usefulness of the MNC assay as a reliable biological indicator for cellular radiosensitivity. While Streffer *et al.* (1982) showed a meaningful correlation between MNC frequency and survival in tumour cells, other reports deny such a

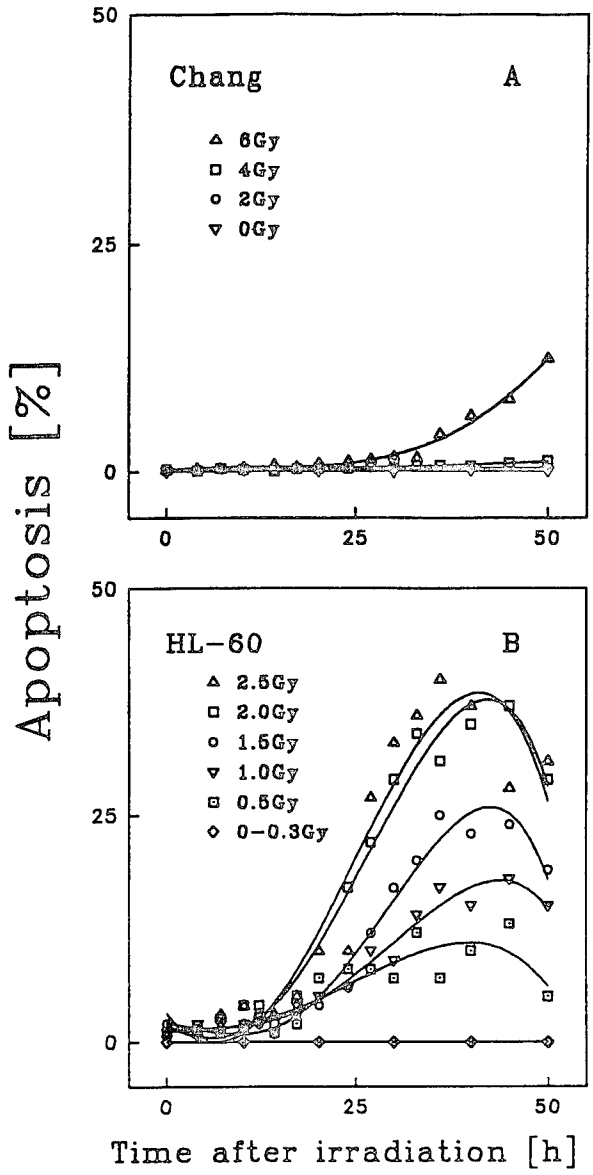


Figure 8. Changes in the number of apoptotic cells after irradiation at different doses for Chang cells (a) and HL-60 cells (b). Typical SEM was $< 0{\cdot}4\%$. Individual polynomial curve fits were performed for each set of data.

correlation. For instance, Bush and McMillan (1993) reported a poor correlation between radiation-induced MNC formation and cell death. In their study, two radioresistant human bladder carcinoma cell lines (MGH-U1 and RT112) exhibited unexpectedly enhanced MNC yields, while two radiosensitive human cell lines, medulloblastoma (D283MED) and neuroblastoma (HX142), showed unexpectedly reduced MNC yields.

In our present study, a similarly poor correlation between radiation-induced MNC% yields and radiosensitivity was observed when comparing the relatively radiosensitive HL-60 cell line with the relatively radioresistant L929 cell line (Figures 1–3). Maximum micronuclei yields were determined in this study by time-course measurements of micronuclei induction following exposure to ionizing radiation (see the review by Almassay *et al.* 1987). This approach was selected over the CB-block technique, an alternative method used to address the cytokinesis problem associated with this cytogenetic assay. The CB-block approach has recently been criticized for several reasons. CB reportedly interferes with actin polymerization, thus preventing the cellular budding that leads to the formation of apoptotic bodies (Kerr *et al.* 1994). In addition, CB is typically dissolved in the solvent dimethylsulphoxide, which has been demonstrated to cause undesired differentiation processes in HL-60 cells (Fuhrmann *et al.* 1992). Furthermore, there is ongoing debate about whether CB induces chromosome damage (Masunaga *et al.* 1990) or influences drug- or radiation-induced MNC frequency in peripheral lymphocytes (Yager *et al.* 1988) and in some tumour cells (Shibamoto *et al.* 1991).

Are there limits on the current use of the micronucleus assay? The effect of combined radiation and hyperthermia treatment on micronuclei induction in human melanoma cells further showed an uncoupling of the predictor ability of MNC yields and cell death (van Beuningen *et al.* 1981). In this earlier study, we demonstrated no difference in MNC induction in cells treated with either X-irradiation dose > 4 Gy or with hyperthermia plus X-irradiation. However, hyperthermia significantly enhanced radiation-induced cell killing. These data suggest an apparent upper limit for the use of the MNC assay as a biological indicator in a combined modality of cancer therapy. This limit is likely due to differing mechanisms of cell death by radiation and hyperthermia.

The potential existence of an upper limit in radiation-induced MNC yields is further supported by our MNC% and MC% findings, illustrated for Chang cells (dose > 4 Gy) and HL-60 cells (dose > 1 Gy) (Figures 2–5). Similar saturation of MNC yields was recently demonstrated with human lymphocytes exposed to neutrons (Wuttke *et al.* 1994). Hence, based on findings using certain tumours and tumour cell lines and using presently available methodologies, it is likely that the detection of the MNC frequency alone is sometimes insufficient to measure radiation-induced injury, making this assay unreliable as a general predictor assay for intrinsic cellular radiosensitivity.

Because in certain tumours apoptosis seems to play an important role in cell death after radiation therapy (Sen 1992, Meyn *et al.* 1993, Stephens *et al.* 1993, Ashwell *et al.* 1994, Gorczyca *et al.* 1994), we also measured, in parallel with MNC, the yield of apoptotic events. While we measured the apoptotic process in

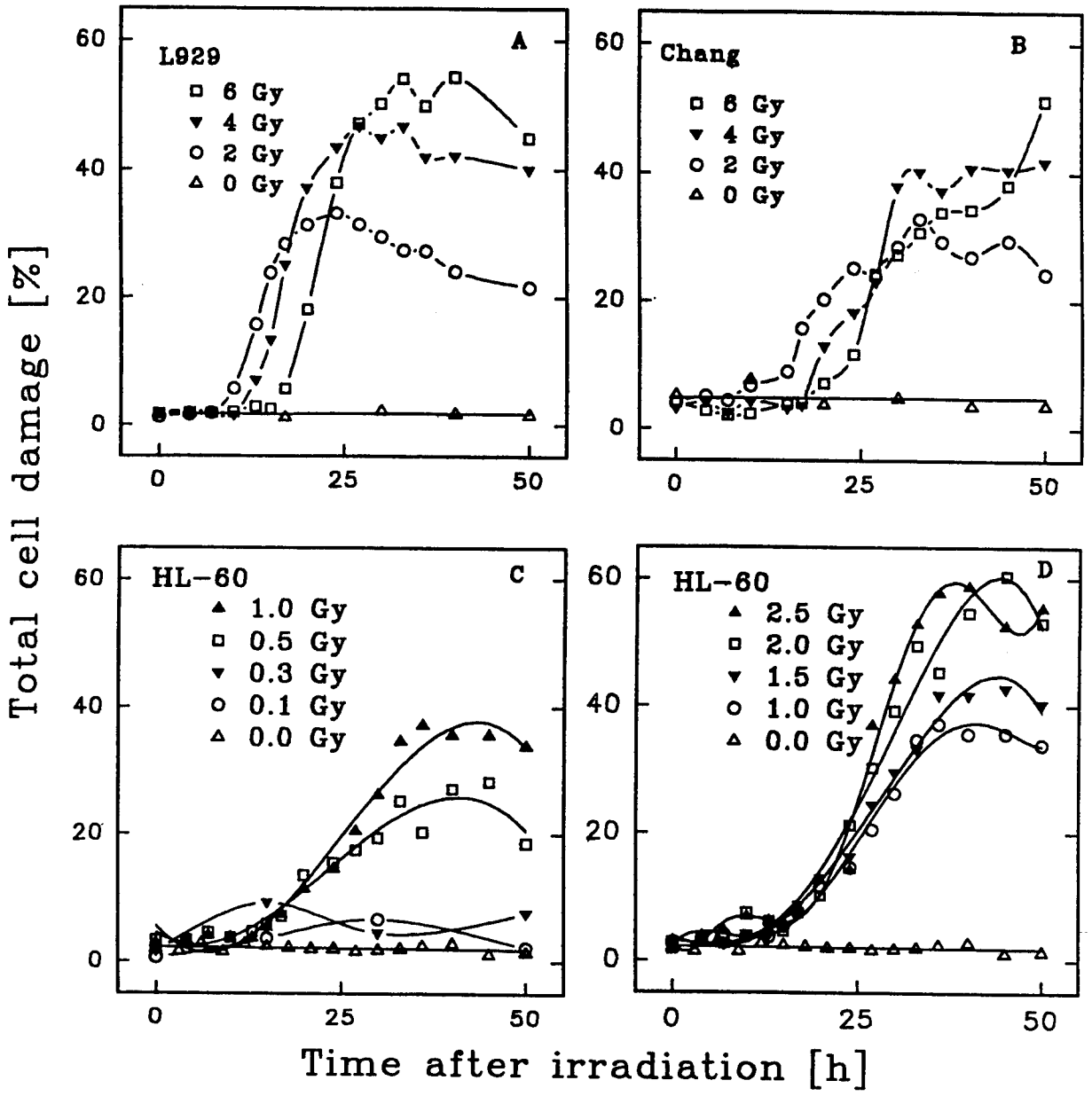


Figure 9. Comparison of the radiation-induced TCD% versus time after irradiation at different doses for L929 cells (a), Chang cells (b) and HL-60 cells (c and d). Typical SEM was < 1·5%. The curves in a and b were obtained by a spline fit connecting sequential data points. The curves in c and d were determined using a polynomial fit through the data points. No process of saturation was apparent in the analysis of the survival/TCD% relationship in any of the three cell lines, in contrast with that of the survival/MNC% (Figure 2b) and survival/MC% (Figure 4b) relationships.

HL-60 cells by both morphological (Figure 6) and electrophoretical (Figure 7) techniques, we were not successful in demonstrating DNA fragmentation in Chang cells by the formation of a classic DNA ladder by the electrophoresis-based method. However, it has been shown by others (Schwartz *et al.* 1993, Vaux 1993) that the DNA ladder can be absent in certain cases of programmed cell death. Our findings with Chang cells were consistent with this phenomenon.

Could both modes of cell death occur in cells exposed to ionizing radiation? The present study, which used mouse fibrosarcoma (L929), human myeloid leukaemia (HL-60), and human epithelial like liver cell lines (Chang), demonstrated varied capacities for radiation-induced apoptosis. For example, as expected, the leukaemia cell line (HL-60) showed the highest dose-dependent yield, while the fibrosarcoma line (L929) showed negligible evidence of apoptosis. This observation generally agrees with previously reported studies. It is interesting to note that, while we showed that biologically significant doses of X-rays fail to induce apoptosis in L929 cells, others have demonstrated

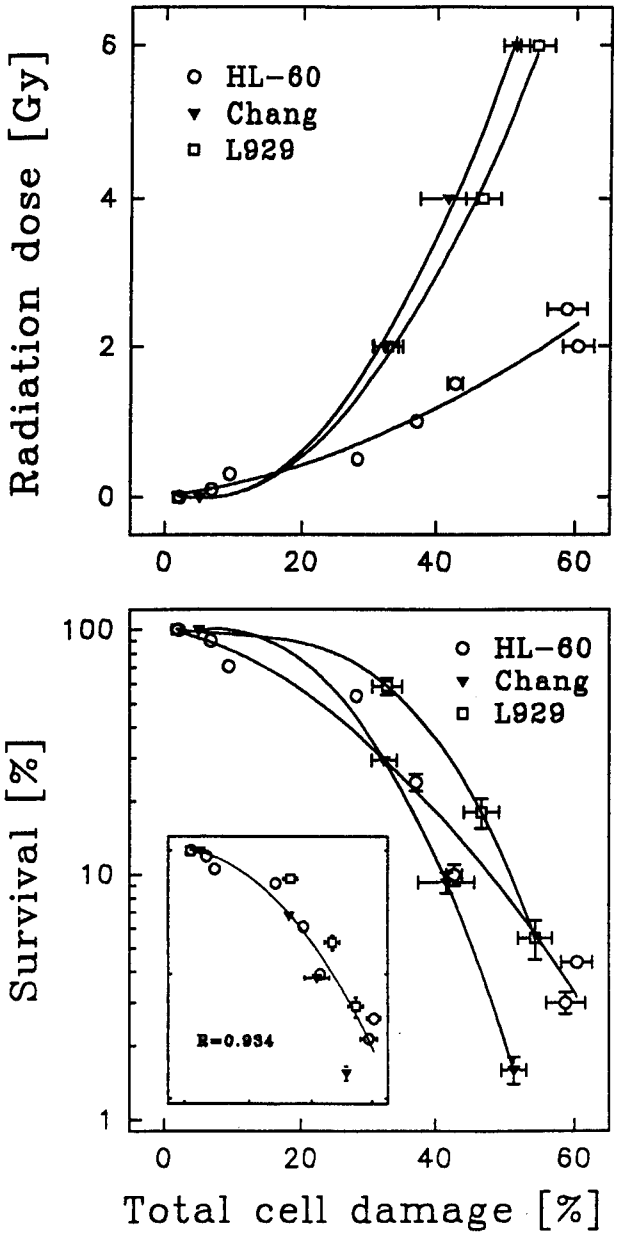


Figure 10. Correlation of the TCD% with radiation dose and survival. The upper and lower graphs represent individual polynomial curve fits of three cell lines examined (with $p < 0{\cdot}05$). SEM of TCD% data (x-bars) and SEM of survival (y-bars, lower graph) are visible when greater than the symbols. The inset displays a cell line-independent, linear–quadratic curve fit through all data points of the cell lines even above the area of saturation, which appears when counting only MNC% or MC% ($\log f(x) = 2{\cdot}0114 - 0{\cdot}0036x - 0{\cdot}000386x^2$; $R = 0{\cdot}934$;$p < 0{\cdot}05$, with '$\log f(X)$', which represents the survival rate in percent, expressed as log to the base 10, and 'x', which represents TCD%). The labels of the ticks in the inset correspond to the labels of the major ticks of the lower graph of Figure 10.

apoptosis in this cell line using benzylideneascorbate (Tanuma *et al.* 1993) and tumour necrosis factor (Fehsel *et al.* 1991). This difference could be due to variations in L929 subclone cell lines, the mechanism of the damaging insult, or unknown factors. However, our findings, using the myeloid leukaemia HL-60 cell line, demonstrate significant micronuclei- and apoptotic-mediated processes, indicating that both modes of cell death can occur in the same cell line. Clearly, more damaging insults and cell lines of various tissue origins (Yamamoto *et al.* 1993, Radford 1994, Radford *et al.* 1994) need to be examined to understand better the importance of apoptosis versus micronucleation events in cell death.

While micronuclei and apoptosis are commonly associated with cell death, their mechanism of action are quite different. Micronuclei are generally accepted to be linked with reproductive cell death following exposure to ionizing radiation (Joshi *et al.* 1982). Chromosome breakage combined with nuclear division events are intimately associated with the process of the appearance of micronuclei in cytoplasm. In contrast, the mechanisms of apoptosis apparently involves a metabolic-and enzymatic-dependent process of abundant DNA break induction. Tauchi and Sawada (1994) recently reported that apoptosis is the major cause of cell death by mitotic failure in a mouse leukaemia cell line (L5178Y). The rationale of introducing the TCD assay as a new predictor assay is based on measuring both mechanisms of cell death in order to obtain a more reliable assessment of the cell survival fraction after radiation exposures. Again, our findings of a common cell line-independent prediction of radiation-induced cell killing, based on the TDC assay (Figure 10, inset) using three cell lines, are consistent with the potential general use of this assay as an improved predictor of radiation injury. Nevertheless, this assay requires several measurements because of the time dependence of the yields of damaged cells, which is of disadvantage. Otherwise, when alternatively using the CB-block method, preliminary experiments are necessary in order to evaluate the correct concentration of the drug for each individual cell line/tumour. Hence, assays that try to predict radiosensitivity still require a considerable amount of measurements nowadays. However, depending on the predominant mode of cell death (e.g. micronucleation in L929 up to a dose of 6 Gy) it may be possible still to use a single assay (e.g. MNC assay for L929) as the endpoint of radiosensitivity of this cell line as long as no saturation of micronucleation occurs. (see above).

In conclusion, the MNC assay (MNC%, MC%) demonstrates limited general use as a predictor assay due to cell-dependent effects of saturations of MNC frequency with increasing radiation dose. However, the TCD assay, which is based on the sum of micronucleated and apoptotic cells, caused a significant cell type-independent correlation with survival over a broad

dose range covering two decades of cell killing. This implies that apoptosis and micronucleation represent the major mechanisms of radiation-induced cell lethality in the cell lines examined. In other words, these data stress the inherent weakness of one assay if applied to cell lines characterized by a different mode of cell killing. Therefore, we recommend consideration of the TCD assay as an improved alternative predictor assay for cellular radiosensitivity, especially in tumours characterized by the occurrence of apoptotic cells.

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Contrasting Mechanisms of the Myeloprotective Effects of Interleukin-1 against Ionizing Radiation and Cytotoxic 5-Fluorouracil

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Neta, R., Keller, J. R., Ali, N., Blanchette, F. and Dubois, C. M. Contrasting Mechanisms of the Myeloprotective Effects of Interleukin-1 against Ionizing Radiation and Cytotoxic 5-Fluorouracil. *Radiat. Res.* **145**, 624–631 (1996).

Pretreatment with a single dose of interleukin-1 (IL-1) counteracts the myelosuppressive effects of radiation. In contrast, multiple doses are required to protect against several cytoablative drugs, suggesting different mechanisms. We examined the possibility that myeloprotection is due to IL-1-induced cycling of primitive progenitor cells. First, we evaluated the effect of the time between administration of IL-1 and 5-fluorouracil (5-FU), which kills cycling cells but spares quiescent early progenitors, on their interaction. Pretreatment with a single dose of IL-1 resulted in the death of mice treated with 5-FU provided IL-1 was given 18 h, but not 4 or 48 h, prior to administration of sublethal doses of 5-FU. Second, evaluation of primitive hematopoietic progenitor cells, 13-day spleen colony-forming units (CFU-S) and CFU with high proliferative potential revealed that treatment with 5-FU 18 h after administration of IL-1 results in reduction of CFU-S by 98% and of CFU with high proliferative potential by 65%, but only a 7 and 10% reduction, respectively, at 48 h. Third, in contrast to protection from death by pretreatment with a single dose of IL-1 at 24 h, two injections of IL-1 at 72 and 24 h before irradiation abrogated such protection. Similarly, the toxicity of 5-FU to progenitor cells was reduced when two injections of IL-1 were administered 48 h apart. This correlates with the time of up-regulation in the bone marrow cells of TGF-β. These findings suggest that, depending on the schedule of treatment, administration of IL-1 may result in cycling of primitive progenitors, to protect against radiation, and may cause inhibition of cycling to protect against chemotherapeutic drugs. © 1996 by Radiation Research Society

INTRODUCTION

Interleukin-1 (IL-1)[2] protects the hematopoietic system of mice against a wide range of cytoablative therapies. For example, pretreatment with IL-1 protects against ionizing radiation (*1*) as well as against drugs such as cyclophosphamide, 5-fluorouracil (5-FU), mafosamide and doxorubicin (*2–4*). However, to achieve such protection, the schedule of administration of IL-1 was varied; i.e., administration of a single dose of IL-1 within 18 to 24 h prior to irradiation was necessary for radioprotection (*1*), whereas administration of multiple daily doses of IL-1 (7 days) was necessary to protect against chemotherapeutic drugs (*2–4*). It is not known why such different treatment regimens are required to achieve protection of the myeloid cells.

Interleukin-1 has a broad range of biological effects, including potent *in vivo* stimulation of hematopoiesis (*5, 6*). Specifically, IL-1 has been shown to synergize with hematopoietic growth factors in promoting the proliferation of stem and progenitor cells (*6–8*). These effects are amplified by the capacity of IL-1 to induce the production of such hematopoietic growth factors (*9–13*) and to up-regulate their receptors on bone marrow progenitors (*14*). Earlier studies found that there was an increase in the number of hematopoietic progenitors in the bone marrow several hours after administration of IL-1 that reached a maximum after 48 h (*14–16*). These findings suggested that an expansion of progenitor cells was the basis for the myeloprotective action of IL-1. However, mice receiving irradiation 48 h after treatment with IL-1 were not protected from death (*17*), indicating that the mere increase in the numbers of progenitor cells was not sufficient for radioprotection by IL-1.

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[2] Abbreviations used: CFU, colony-forming unit; CFU-C, CFU in culture; CFU-S, spleen colony-forming unit; 5-FU, 5-fluorouracil; G-CSF, granulocyte colony-stimulating factor; HU, hydroxyurea; IL-1, interleukin-1; IMDM, Iscove's modified Dulbecco's medium; TGF-β, transforming growth factor β.

In view of these observations, we hypothesized that the radioprotective effect of IL-1 may be attributed to the cycling of progenitor cells and to the increased radioresistance of the cells in the late S phase of the cell cycle, as suggested by earlier studies (*18–20*). Indeed, we have demonstrated that administration of IL-1 18 h prior to irradiation, which resulted in optimal radioprotection, coincided with increased sensitivity of progenitor cells of various lineages to hydroxyurea (HU) (*21, 22*). Since HU is selectively toxic for cells in the S phase, these results implied that IL-1 induced progenitor cells to progress to the S phase.

Since bone marrow progenitor cells giving rise to spleen colony-forming units (CFU-S) or CFU with high proliferative potential represent 0.01% of the total bone marrow population, isolation of these cells in numbers sufficient for direct flow cytometry of the cell cycle status (i.e. 10^5 cells/assay, which would require 100 femurs) would be prohibitive. Instead, to test the above hypothesis, we studied the effect of 5-FU on bone marrow cells of mice treated with IL-1. 5-Fluorouracil is preferentially toxic to cycling cells and is used to enrich the fraction of early progenitor cells in the bone marrow (*23–25*). Such enrichment is based on the relatively quiescent status of the stem cell populations. Thus we hypothesized that, if IL-1 induces more primitive progenitors to cycle, these cells would become sensitive to 5-FU. In this report we demonstrate that IL-1 given in a radioprotective regimen sensitizes progenitor cells to 5-FU, suggesting that myeloprotection is associated with cycling of primitive hematopoietic progenitors, whereas repeated doses of IL-1 abrogate radioprotection and cycling of the same progenitor cells. These results imply that myeloprotection against radiation and against chemotherapy is based on contrasting mechanisms.

MATERIALS AND METHODS

Mice

$B6D2F_1$ female mice, 8–10 weeks old, were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were handled as described previously (*1*).

Treatment

Recombinant human interleukin-1 (rhIL-1α 117-271 Ro 24-5008, lot IL-1 2/88, activity 3×10^8 U/mg) was kindly provided by Dr. Peter Lomedico, Hoffmann-La Roche (Nutley, NJ). Interleukin-1 was diluted in pyrogen-free saline on the day of injection and was given intraperitoneally (ip) at a concentration of 0.2 ml/mouse. 5-Fluorouracil (SoloPak, IL) was injected intravenously (iv) at concentrations ranging from 150 to 500 mg/kg.

Irradiation

Mice were randomized, placed in ventilated Plexiglas containers and bilaterally irradiated using the AFRRI ^{60}Co whole-body irradiator as specified (*1*).

Hematopoietic Colony-Forming Assays

Determinations of CFU-S were carried out as described by Till and McCulloch (*26*). Recipient mice were exposed to 9.5 Gy whole-body ^{60}Co irradiation. This dose was sufficient to reduce background macroscopic colonies at 8 days to less than one per spleen. Bone marrow cell suspensions were diluted to 5×10^4 to 2×10^5 cells in 0.2 ml and injected into the caudal vein of each recipient mouse. For each bone marrow cell suspension two groups of recipient mice, four mice/group, received concentrations of bone marrow cells that varied twofold. After 9 and 13 days spleens were removed and placed into Bouin's fixative, and the macroscopic colonies were counted.

For determinations of CFU in culture (CFU-C) and CFU with high proliferative potential, bone marrow cells were resuspended in Iscove's Modified Dulbecco's Medium (IMDM) with 10% FCS, 1% L-glutamine and antibiotics and plated in 0.3% Seaplaque agarose (FMC Bioproducts, Rockland, ME) in 35-mm Lux petri dishes (Miles Laboratory Inc., Naperville, IL). Cultures were supplemented with predetermined optimal doses of stem cell factor (SCF), IL-3, IL-11 and granulocyte colony-stimulating factor (G-CSF). Dishes were incubated in a fully humidified atmosphere at 37°C in 5% CO_2 for 7 to 10 days before the growth of CFU-C was scored. Colony-forming units with high proliferative potential were generated in a double-layer agar culture system consisting of a 1-ml underlayer of α-modified Eagle's medium (α-MEM) with 20% FBS, 0.5% Seaplaque agarose plus growth factors (specified above) and a 0.5-ml overlayer of α-MEM containing 20% FBS, 0.3% Seaplaque agarose and bone marrow cells. The plates were scored for the formation of colonies greater than 0.75 mm in diameter after 14 days of incubation at 37°C in 5% O_2, 10% CO_2 and 85% N_2.

Determination of TGF-β mRNA

Plasmids and probes. The rat TGF-β1 cRNA riboprobe was generated from the 984 nts cDNA clone obtained from the American Type Culture Collection (Gene Bank/EMBL:X52498). The clone corresponds to the rat TGF-β1 cDNA described previously (coding region 413–1582) (*27*). The vector was linearized with *Sma* I and the cRNA probe was 850 nts. Radiolabeled riboprobes were prepared using [^{32}P]UTP (29.6 TBq/mmol; Amersham, Arlington Heights, IL) according to the Ambion MAXIscript™ *in vitro* transcription kit (Ambion, Austin, TX). Briefly, transcription mixtures contained 1.85 MBq [^{32}P]UTP; 10 m*M* DTT; 0.5 m*M* ATP, CTP and GTP; 1× transcription buffer; 12.5 U RNase inhibitor; 1 μg of the appropriate linearized plasmid; and T7 RNA polymerase in a total volume of 20 μl. The reaction was carried out for 90–120 min at 37°C.

Northern analysis. Total RNA was extracted from mouse bone marrow cells according to the Tri-Reagent protocol (Molecular Research Center Inc., Cincinnati, OH) (*28*) described previously. Aliquots of 5 μg of total RNA were run on a horizontal gel apparatus in 1% agarose gel containing 1× MOPS and 6% formaldehyde submerged in 1× MOPS buffer (pH 7.0). The samples were transferred onto a nylon membrane Hybond N+ (Amersham) by overnight capillary action with 10× SSC. After blotting, the RNA was fixed with 0.05*N* NaOH and the membranes were stained in 0.02% methylene blue and 0.3 *M* sodium acetate (pH 5.5). The membranes were then prehybridized for 2 h at 68°C with 1× hybridization buffer containing 120 m*M* Tris (pH 7.4), 600 m*M* NaCl, 8 m*M* EDTA (pH 8.0), 0.1% Na_4PP_i, 0.2% SDS, 62.5 μg/ml heparin and 10% dextran sulfate. Hybridization began with the addition of the [^{32}P]UTP-labeled cRNA probe and carried out overnight in one part 2× hybridization buffer and one part formamide. The membranes were washed sequentially in 2× SSC/1% SDS at room temperature, 2× SSC/1% SDS at 68°C, 0.1× SSC/0.2% SDS at 68°C and 0.1× SSC/0.1% SDS at 68°C and exposed to X-ray film with intensifying screens at –80°C for times ranging from 6 h to 3 days.

Determination of bioactive TGF-β. Total bone marrow cells from mice treated with saline or IL-1 were incubated at 3×10^6 cells/ml in IMDM with 0.1% FCS, 1% L-glutamine and antibiotics. Forty-eight hours after incubation, supernatants were collected, heat-activated (80°C, 10 min) and assayed for active TGF-β using a commercially available ELISA kit specific for TGF-β1 (R&D Systems, Minneapolis, MN). The limit of detection for this assay is 30 pg/ml TGF-β.

Statistical Analysis

Data were analyzed for significant differences using one-way ANOVA.

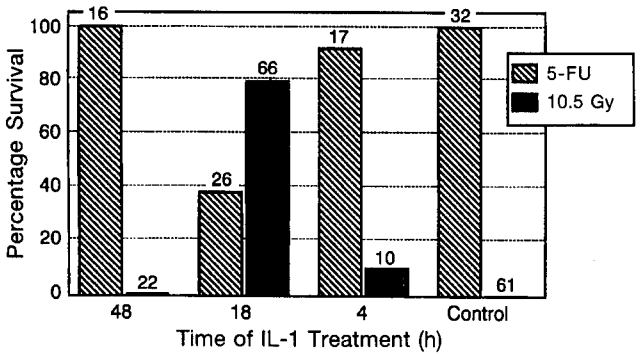

FIG. 1. Converse effects of IL-1 on survival of 5-FU or of γ-irradiated B6D2F$_1$ mice. B6D2F$_1$ mice received 1 μg IL-1/mouse ip at designated times prior to iv injection of 360 mg/kg 5-FU, or exposure to lethal (10.5 Gy) γ radiation. The data represent the percentage of mice that survived for 30 days. In additional experiments 100% of control mice survived after receiving 5-FU iv in doses up to 500 mg/kg. The numbers above the bars indicate the numbers of mice in the experimental group.

RESULTS

Comparison of the Effect of IL-1 on Survival of Irradiated and 5-FU-Treated Mice

In previous studies, a single dose of 1 μg IL-1 per mouse given ip 18 to 24 h prior to lethal irradiation protected a significant fraction of the mice from death. The same treatment given 4 or 48 h prior to irradiation was ineffective (*17*). We examined the effect of IL-1 on mice receiving 5-FU (Fig. 1). Whereas a dose of 500 mg/kg 5-FU was sublethal in control B6D2F$_1$ mice, 62% of mice pretreated with IL-1 18 h prior to a lower dose of 360 mg/kg 5-FU died. In contrast, IL-1 given 48 h prior to 5-FU did not augment the toxicity of this drug. Similarly, the lethal effect was reduced significantly when IL-1 was given 4 h prior to administration of 5-FU. Thus, 18 h after treatment with IL-1, there was an inverse correlation between the protective effect of IL-1 against lethal irradiation and its sensitizing effect on 5-FU toxicity.

Critical Influence of the Interval between Administration of IL-1 and 5-FU on the Numbers of Surviving 9- and 13-Day CFU-S

Previous studies indicated that IL-1 induces expansion of bone marrow progenitors (*14–16*). Although the maximal number of such progenitor cells was detected at 48 h after administration of IL-1, the actual kinetics of their cycling was not examined. The question arises whether the differences in the radioprotective effects of IL-1 at times after its administration (as above) may be consistent with alterations in the cell cycling status of the CFU-S recovered at 9 and 13 days. To address this question, mice were treated with 1 μg IL-1 per mouse 4, 18 and 48 h prior to administration of the dose of 150 mg 5-FU per mouse usually employed. Femoral bone marrow cells were harvested 4 days after administration of 5-FU and injected into lethally irradiated (9.5 Gy) recipients. The splenic colonies in the recipient mice were scored at 9 and 13 days. The results (Table I) show that treatment with IL-1 18 h prior to administration of 5-FU resulted in a considerable reduction in the numbers of CFU-S compared to mice given only 5-FU (99.7 and 98% for 9- and 13-day colonies, respectively), suggesting that treatment with IL-1 resulted in cycling of these cells. Although the reduction in the CFU-S was also observed when 5-FU was administered 48 h after IL-1, the numbers of CFU-S per femur surviving such treatment were >200 times greater than those surviving 18 h after IL-1 for 9-day CFU-S (75% compared to 0.3%), and >40 times greater for 13-day CFU-S (93% compared to 2%). Similarly, the numbers of CFU-S when there was a 4-h interval between administration of IL-1 and 5-FU were significantly greater than those at 18 h (10% compared to 0.3% for 9-day colonies and 17% compared to 2% for 13-day colonies).

The bone marrow cells from mice given IL-1 and 5-FU at 18 h were also grown in soft agar containing IL-3 plus SCF or IL-3 plus SCF, IL-11 and G-CSF (Table II). Although the numbers of both CFU-C and CFU with high proliferative

TABLE I
The Effect of the Interval between Administration of IL-1 and 5-FU on the Numbers of CFU-S/Femur

Treatment	4 h		18 h		48 h	
	Number	Percentage	Number	Percentage	Number	Percentage
			CFU-S (9 days)[a]			
Saline + 5-FU	1597 ± 687	100	1320 ± 209	100	1805 ± 346	100
IL-1 + 5-FU	155 ± 58*	10	4 ± 4*	0.3	1348 ± 284	75
			CFU-S (13 days)			
Saline + 5-FU	3340 ± 777	100	2688 ± 466	100	2306 ± 345	100
IL-1 + 5-FU	578 ± 153*	17	58 ± 21*	2	2157 ± 181	93

Notes. Donor B6D2F$_1$ mice (three mice/group) were given ip saline or 1 μg/mouse of IL-1 and at specified times 5-FU 150 mg/kg, iv. Four days later femoral bone marrow cells were injected to lethally (9.5 Gy) irradiated recipients (four mice/group). The results are the mean of two separate experiments ± SD. In all cases the number of colonies at 18 h is significantly smaller ($P < 0.01$) than that at 4 h.

[a]Days after completion of treatment.

*$P < 0.01$ compared to saline + 5-FU.

TABLE II
The Effect of the Interval between Administration of IL-1 and 5-FU on the Numbers of CFU-C/Femur and CFU with High Proliferative Potential/Femur

	IL-3 + SCF				IL-3 + SCF + IL-11 + G-CSF			
	CFU-C		CFU with high proliferative potential		CFU-C		CFU with high proliferative potential	
Treatment	Number	Percentage	Number	Percentage	Number	Percentage	Number	Percentage
Saline + 5-FU	6443 ± 697	(100)	1655 ± 455	(100)	6339 ± 1503	(100)	1725 ± 515	(100)
IL-1 (18 h) + 5-FU	1090 ± 113*	(17)	602 ± 38*	(36)	1504 ± 382*	(24)	602 ± 38*	(35)
IL-1 (48 h) + 5-FU	6610 ± 3110	(102)	1366 ± 389	(82)	7776 ± 3888	(123)	1555 ± 778	(90)

Notes. The bone marrow cells from donor mice treated as described in Table I were grown as described in Materials and Methods. The results are the mean of two separate experiments ± SD.
*$P < 0.01$.

potential were reduced significantly in mice given IL-1 18 h prior to 5-FU compared to control mice receiving only 5-FU, the reduction was only 75% for CFU-C and about 65% for CFU with high proliferative potential. Furthermore, IL-1 given 48 h prior to 5-FU did not affect the numbers of CFU-C and CFU with high proliferative potential significantly. Thus, while there were no major differences in the percentages of CFU with high proliferative potential and CFU-S (13 days) that were sensitive to 5-FU at 48 h after administration of IL-1, there were 10-fold more CFU-S than CFU with high proliferative potential sensitive to 5-FU at 18 h after administration of IL-1. Therefore, sensitivity to 5-FU at 18 h after treatment with IL-1 is much more pronounced for the cells giving rise to splenic colonies than for the more primitive colonies grown in culture medium.

Effect of 48-h Pretreatment with IL-1 on the Susceptibility of CFU-S to Subsequent Treatment with IL-1

The reduced susceptibility of the CFU-S to 5-FU at 48 h after administration of IL-1 may be due to either transient effects of IL-1 due to its short half-life or tachyphylaxis, i.e. inability of the CFU-S to respond to further stimulus by IL-1. To assess the latter possibility, groups of mice received either two injections of IL-1 48 h apart or a single injection of IL-1, in both cases followed by 5-FU 18 h later. The bone marrow from such mice and control mice given only 5-FU was examined for CFU-S in lethally irradiated recipients. The results in Table III show that the numbers of CFU-S in mice treated with one dose of IL-1 were 2.5% of the control, whereas with two doses of IL-1 they were 25% of the control. This result indicates that the first of the two treatments with IL-1 renders 22.5% of progenitor cells refractory to sensitization to 5-FU by the second IL-1 treatment.

Effect of Pretreatment with IL-1, 48 h Prior to a Second IL-1 Injection, on Radioprotection

To assess whether the reduced ability of progenitor cells to respond to a second injection of IL-1 affects the ability of mice to survive exposure to ionizing radiation, groups of mice were treated with (a) a single dose of IL-1 24 h prior to irradiation or (b) two injections at 48-h intervals, i.e. at 72 and 24 h prior to irradiation. Whereas 100% of mice receiving one injection of IL-1 within 24 h prior to irradiation survived radiation exposure, mice receiving two injections 48 h apart were no longer protected from radiation lethality (Table IV). Thus the inability of CFU-S to become sensitized to 5-FU lethality was associated with abrogation of the radioprotective effect of IL-1 18 h prior to irradiation.

Effect of IL-1 Pretreatment on TGF-β mRNA Expression and Protein Production by Bone Marrow Cells

Transforming growth factor β is a potent radiosensitizer (*29*) and inhibitor of the cell cycle in various cell types,

TABLE III
The Effect of Pretreatment with Two Injections of IL-1 Prior to 5-FU on the Numbers of CFU-S/Femur (9 days)

Treatment	Bone marrow cells/femur[a]	CFU-S/femur	Percentage
Saline	2.8×10^6	580 ± 56	100
IL-1 (one injection)	1.1×10^6	15 ± 5	2.5
IL-1 (two injections)	1.7×10^6	145 ± 32	25

Notes. Donor mice (three mice/group) were given two injections of 1 μg/mouse of IL-1 48 h apart at 72 and 24 h prior to 5-FU, one injection of IL-1 at 24 h prior to 5-FU, or saline injection 24 h prior to 5-FU.
[a]Cells obtained from femurs of donor mice.

TABLE IV
Effect of Different Schedules of Administration of IL-1 on Survival of Mice after 9.5 Gy Irradiation

Treatment	Dead mice/total mice	Percentage survival
Saline	30/35	16
IL-1 (−72 h; −24 h)	14/17	18
IL-1 (−24 h)	0/20	100

Notes. Mice received 1 μg/mouse of IL-1 at times as specified before lethal irradiation. The results represent mice surviving for 30 days.

TABLE V
Kinetics of Induction of TGF-β by IL-1

Treatment	TGF-β (ng/10^7 cells)	Percentage increase
Saline	0.64	—
IL-1 (1 h)	0.66	3
IL-1 (2 h)	0.71	11
IL-1 (6 h)	1.04	62.5
IL-1 (24 h)	1.20	87.5
IL-1 (48 h)	1.60	150

Notes. Bone marrow cells from mice treated with saline or IL-1 were incubated at 3×10^6 cells/ml for 48 h. Supernatants were collected, heat-activated (80°C for 10 min) and assayed for TGF-β1 as described in Materials and Methods. Each group represents a pool of bone marrow cells from three mice.

including bone marrow progenitors (*30, 31*). In this regard we asked whether administration of IL-1 modulates the expression of TGF-β. Total bone marrow cells from mice treated with saline or IL-1 were cultured, supernatants were harvested at 48 h and heat-activated, and TGF-β was measured using a specific ELISA. Data in Table V indicate that administration of IL-1 to mice results in a time-dependent increase in TGF-β1. To determine whether the observed increase in TGF-β was a consequence of increased TGF-β mRNA expression, Northern blot analysis of total cellular RNA from mice treated with saline and IL-1 was performed. Autoradiography of the membrane showed a 2.5 kb signal upon probing with a TGF-β1 RNA probe (Fig. 2). Treatment with IL-1 resulted in a time-dependent increase in TGF-β1 mRNA, first detected at 30 h after treatment and reaching a 1.65-fold increase at 48 h.

DISCUSSION

This study sheds light on the seemingly paradoxical finding that IL-1 induces cycling of progenitor cells (*21, 22*) and yet protects bone marrow cells from chemotherapeutic drugs that killed cycling cells (*2–4*). There was a 50-fold reduction of 13-day CFU-S in mice that received IL-1 18 h prior to 5-FU compared to mice treated with 5-FU alone. Since 5-FU is toxic primarily to cycling cells, this suggests that IL-1 induced 98% of the normally 5-FU-resistant, slowly proliferating or resting cells to cycle. This finding contrasts with the reduction of less than 3-fold in the numbers of CFU with high proliferative potential and 4-fold in the numbers of CFU-C, both grown with the supply of hematopoietic growth factors. Such differences in the sensitivity of these cells to 5-FU after treatment with IL-1 may be based on several mechanisms: (a) CFU with high proliferative potential are more primitive than 13-day CFU-S (*32*), and fewer cells that form CFU with high proliferative potential cycle in response to IL-1 and are therefore less sensitive to 5-FU; and/or (b) since both types of colonies are grown in culture, an optimal supply of hematopoietic growth factors, as opposed to the case of lethally irradiated recipients, may allow for more effective repair and prevent apoptosis. Nevertheless, 65% of cells forming colonies with high proliferative potential were sensitive to 5-FU at 18 h, suggesting that within this time frame IL-1 induces a majority of these normally quiescent cells to cycle. In contrast, only 10% of CFU with high proliferative potential remained susceptible to 5-FU 48 h after treatment with IL-1.

The kinetics of susceptibility of 13-day CFU-S to 5-FU at 4, 18 and 48 h after treatment with IL-1 (83, 98 and 7% of control, respectively) suggests that the majority of these cells enter the cell cycle within 4 h and most of them have ceased to cycle 48 h after treatment with IL-1. The lack of significant radioprotection at 4 h after administration of IL-1 compared to optimal protection at 18 to 24 h supports the hypothesis that reaching the S phase is critical for radioprotection. This was supported further by the finding that the absence of radioprotection 48 h after treatment with

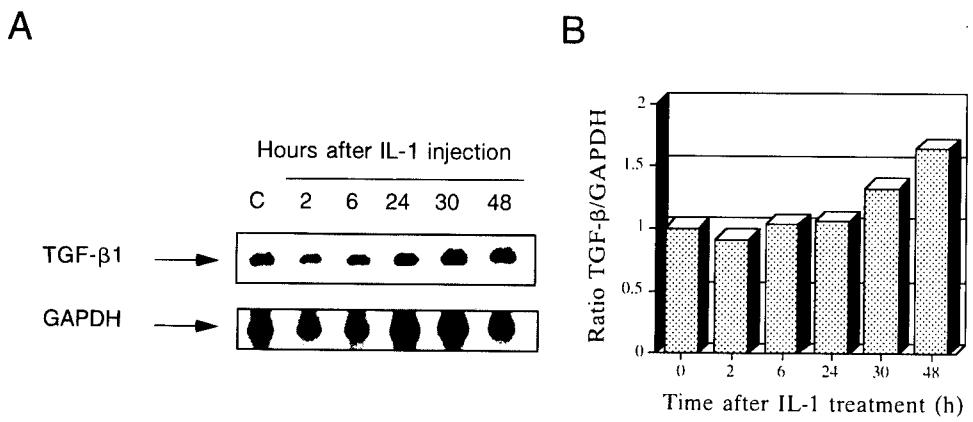

FIG. 2. Effect of administration of IL-1 on TGF-β1 mRNA levels in bone marrow cells. Bone marrow cells from mice treated with saline or IL-1 were harvested, and total cellular RNA was extracted and hybridized with a rat TGF-β1 cRNA probe and GAPDH cDNA probe as described in Materials and Methods. Densitometric values are expressed as ratios of rat TGF-β1 mRNA/GAPDH densitometry quantification with control values set at 1. Panel A: Northern blot autoradiogram; panel B: densitometric analysis of the corresponding autoradiogram.

IL-1 correlated with only 7–10% of the early progenitors remaining in cycle (Tables I and II).

The demonstration that the 48-h interval between the two injections of IL-1 is associated with the loss of susceptibility to 5-FU by a substantial fraction of CFU-S (Table III) and prevents radioprotection (Table IV) provides an additional new insight into the effects of IL-1. The refractory status of more than 20% of CFU-S to killing by 5-FU in mice that have been treated with two injections of IL-1 at 72 and 24 h prior to administration of 5-FU (Table III) suggests that the 48-h pretreatment with IL-1 resulted in abrogation of the ability of CFU-S to cycle in response to a subsequent IL-1 challenge. One explanation for such an effect may depend on previously observed (*33*) up-regulation and shedding of decoy-like Type II IL-1R that interacts with IL-1, resulting in tachyphylaxis. Moreover, we observed previously that TGF-β sensitized mice to radiation lethality (*29*). It was therefore of interest to examine the potential involvement of TGF-β, in particular, since the induction of TGF-β by IL-1 in the bone marrow has not been reported previously. In fact, the inability of pretreatment with IL-1 at 48 h to render the progenitor cells susceptible to 5-FU toxicity correlates in time with up-regulation of the production of TGF-β1 by bone marrow cells to 1.6 ng/10^7 cells at 48 h after administration of IL-1 (Table V).

Transforming growth factor β has been reported to be a potent inhibitor of the cell cycle for many cell types (*30, 31*), acting through the reduction of the expression of multiple growth factor receptors, including c-kit (*34–40*), production of Kit ligand (*41*) and induction or activation of cell cycle inhibitors (*42, 43*). Concentrations of TGF-β similar to those released by IL-1-treated bone marrow cells in our studies inhibited the *in vitro* growth of primitive progenitor cells, whereas the growth of more differentiated granulocyte progenitors (G-CFU) was stimulated (*44*). Interestingly, we have observed previously that five daily injections of IL-1 resulted in highly granulocytic bone marrow (Neta, unpublished results). Thus our current results on IL-1-induced up-regulation of TGF-β suggest participation of TGF-β in this effect.

In addition to TGF-β, other factors induced by IL-1 and inhibitory to cycling and proliferation of hematopoietic progenitors may be involved. For example, prostaglandin and TNF, both induced by IL-1, were reported to inhibit the growth of progenitor cells (*45–47*). Repeated injections of IL-1 led to the appearance of a serum factor inhibitory to colony formation that was partially neutralized by antibody to TNF (*45*). More recently, IL-1 was shown first to up-regulate and then to down-regulate production of GM-CSF in human fibroblasts. The down-regulation was associated with the production of prostaglandins (*48*). Our findings suggest, therefore, that a cascade of cytokines and other mediators induced by IL-1 includes positive as well as negative regulators of cycling of primitive progenitors.

Taken together, these results demonstrate for the first time that: (1) Not only is the radioprotective effect of IL-1 dependent on an increase in progenitor cell numbers, but the optimal radioprotective effect of IL-1 (at 18 to 24 h) coincides in time with the highest sensitivity of early progenitor cells to 5-FU, suggesting their cycling. (2) Forty-eight hours after administration of IL-1 a large fraction of early progenitor cells are no longer susceptible to 5-FU lethality, suggesting that these cells no longer cycle, concomitant with increased production of TGF-β in the bone marrow. (3) The abrogation of the ability of IL-1 to render progenitor cells susceptible to 5-FU in response to a secondary IL-1 treatment is associated with abrogation of the radioprotective effect of IL-1.

In contrast to chemoprotection achieved by administration of multiple doses of IL-1, five daily injections of IL-1 increased the sensitivity of mice to ionizing radiation (Neta, unpublished results). Together, these findings suggest that the myeloprotective effects of IL-1 against ionizing radiation and cytotoxic chemotherapeutic drugs are mediated by distinct mechanisms based on either induction of cell cycle or inhibition of cycling of progenitor cells.

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Ebselen Inhibition of Apoptosis by Reduction of Peroxides

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ABSTRACT. We investigated the capacity of ebselen [2-phenyl-1,2-benzisoselenazol-3(2*H*)-one], a glutathione peroxidase mimic, to protect cells from radiation-induced apoptosis. Incubating mouse thymocytes with 25 μM ebselen immediately after ^{60}Co gamma-radiation exposure (5 Gy) inhibited morphological changes associated with apoptosis. Treatment of thymocytes with ebselen before, during, or after irradiation completely blocked internucleosomal DNA fragmentation, a biochemical marker for apoptosis. We measured peroxides formed in cells during and after irradiation, using the oxidation-sensitive fluorescent probe 2′,7′-dichlorofluorescin diacetate. By 2 min postirradiation, levels of peroxides in irradiated thymocytes were approximately 10–11 times greater than those in the same cells before irradiation, and levels continued to increase with time. We also measured membrane lipid peroxidation using *cis*-parinaric acid, a naturally fluorescent polyunsaturated fatty acid that readily incorporates into cell membranes. The oxidation of *cis*-parinaric acid also began soon after irradiation and increased with time. Peroxide generation and membrane lipid peroxidation preceded both internucleosomal DNA fragmentation and morphological changes characteristic of apoptosis. Treatment of cells with ebselen reduced peroxide levels and appeared to protect thymocytes from radiation-induced apoptosis by scavenging peroxides generated during and after irradiation. The results suggest that peroxide generation and membrane lipid peroxidation may be important signaling events that trigger apoptosis in irradiated cells. BIOCHEM PHARMACOL 51;11:1443–1451, 1996.

KEY WORDS. apoptosis; ebselen; peroxides; lipid peroxidation; radiation; lymphocytes

Apoptosis is a distinct mode of cell death that has been shown to play a critical role in many aspects of biology and medicine. A wide range of diseases are now thought to be associated with the inappropriate induction of apoptosis, including acquired immune deficiency syndrome and a variety of neurodegenerative disorders [1]. Apoptosis can be triggered experimentally by a variety of extra- and intracellular stimuli, one of which is ionizing radiation [2, 3]. Radiation-induced apoptosis has been characterized primarily in thymocytes, splenocytes, and other cells of hematopoietic lineage that constitute the immune system [4]. Radiation doses as low as 0.05 Gy induce apoptosis in lymphocytes [5].

Most of the cellular damage produced by ionizing radiation is a consequence of the production of free radicals. These highly reactive intermediates can directly react with and alter biological molecules or can produce secondary species that then react. The plasma membrane is especially susceptible to free radical damage because it contains significant quantities of easily peroxidizable lipids and proteins [6, 7]. We have shown previously that membrane lipid peroxidation is an important, early lesion that plays a role in radiation-induced apoptosis [8, 9]. Free radical activity initiated by ionizing radiation in the aqueous extra- and intracellular media adjacent to membranes can be propagated and amplified by lipid peroxidation chain reactions within the membrane [10, 11]. Membrane lipid peroxidation generates hydroperoxides, lipid hydroperoxides, and aldehydes of lipid hydroperoxides, all of which have been shown to be mediators of apoptosis [12, 13].

The cell possesses a number of defense systems that are involved in the protection from free-radical damage. One is the selenium-dependent enzyme glutathione peroxidase, which functions to detoxify peroxides. This protective system, however, can become overwhelmed after exposure to damaging doses of radiation. Ebselen, a relatively non-toxic selenoorganic compound, has been shown to exhibit glutathione peroxidase-like activity that can reduce peroxide levels in many physiological and pathological conditions [14, 15]. Because our earlier work suggested that peroxide production plays an important role in radiation-induced apoptosis, we sought to test if ebselen might prove effective at blocking apoptosis in irradiated cells.

MATERIALS AND METHODS

Thymocyte Incubation Medium

Thymocytes were incubated in either an RPMI-based medium (hereafter referred to as RPMI) or Na^+ Hanks' buffer.

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RPMI consisted of RPMI 1640 medium containing 25 mM HEPES buffer, 2 mM L-glutamine, 55 μM 2-mercaptoethanol, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B (all GIBCO/BRL, Grand Island, NY, U.S.A.), and 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT, U.S.A.). Na^+ Hanks' buffer, pH 7.4, consisted of 10 mM HEPES, 145 mM NaCl, 1.3 mM $MgCl_2$, 1.6 mM $CaCl_2$, 4.5 mM KCl, 0.5 mg/mL bovine serum albumin, and 1.8 mg/mL glucose.

Thymocyte Isolation

CD2F1 male mice, 6 to 7-weeks-old, were euthanized with CO_2. Thymuses were removed aseptically, rinsed in RPMI, and homogenized by pressing in a Stomacher device (Techman Co., Cincinnati, OH, U.S.A.) for 1 min. Single-cell suspensions were prepared by filtering the homogenate through 100 μm nylon mesh. Suspensions were washed once and resuspended in RPMI. Cell numbers were obtained using a Coulter Counter (model ZM, Coulter Industries, Luton, Beds, England), and cell viability was estimated by the capacity of the cells to exclude trypan blue [16].

Ebselen Treatment

Stock solutions (10 mM) of ebselen (Cayman Chemical Co., Ann Arbor, MI, U.S.A.) were prepared in DMSO. Cells were incubated in RPMI containing 25 μM ebselen at 37° in an atmosphere of 5% CO_2 in air before, during, or after irradiation, depending on the experiment. All cell suspensions not treated with ebselen were brought to a concentration of DMSO equivalent to that delivered with ebselen.

Irradiation

Thymocytes were suspended in either RPMI or Na^+ Hanks' buffer (approximately 10^7 cells/mL) and irradiated at room temperature with doses of 5 or 10 Gy at a dose rate of 0.5 Gy/min using the Armed Forces Radiobiology Research Institute's ^{60}Co Facility.

Microscopy

Cells (0.5 to 1 × 10^7) were pelleted by centrifugation (750 *g*, 10 min) and fixed by resuspension in 1 mL of freshly prepared 3% formaldehyde in ice-cold HBSS.* Fixed cells could be maintained under refrigeration for several weeks without any apparent degradation. Cells were concentrated for microscopy by allowing them to settle by gravity to the bottom of a test tube at 4°. Centrifugation to pellet cells was avoided because centrifugation of the fixed cells appeared to contribute to distorted cell morphology. For fluorescence microscopy, all but about 0.1 mL of the fixing buffer overlaying the cells was removed, and the cells were resuspended gently in the remaining buffer. A 20-μL aliquot was removed and mixed with 20 μL of 0.1 mg/mL of ethidium bromide in HBSS (final concentration, 50 μg/mL). The stained suspension was kept in the dark on ice until used. Ten microliters of suspension was placed on a microscope slide and gently covered with a 20-mm square cover slip. The cover slip was sealed with cement to prevent drying. Cells were allowed to settle and adhere to the surface of the slide for 5–10 min before beginning observation. Photomicroscopy was performed with an Olympus AHBT3 Research Microscope with Nomarski-type differential interference contrast and reflected-light fluorescence. Images were preserved on high-speed Polaroid Type 57 film.

DNA Agarose Gel Electrophoresis

Electrophoresis of DNA was performed according to the method of Gong *et al.* [17], which is particularly applicable to the qualitative detection of internucleosomal DNA fragments typical of apoptosis. Briefly, 1–2 × 10^6 cells were pelleted from the medium, washed once with HBSS, resuspended in 1 mL of HBSS, diluted with 10 mL of ice-cold 70% ethanol, and stored at −20° for 24 hr. The cells were then pelleted by centrifugation (800 *g* for 10 min) and ethanol was removed completely. The pellet was resuspended and the cells were lysed in 40 μL of phosphate-citrate buffer (192 parts of 0.2 M Na_2HPO_4 and 8 parts of 0.1 M citric acid, pH 7.8). After incubation at room temperature for 30 min, the cell lysate was centrifuged (1000 *g* for 5 min) and the supernatant concentrated to about 20 μL using a Speed Vac concentrator (Savant Instruments, Farmingdale, NY, U.S.A.). A 3-μL aliquot of 0.25% Nonidet P-40 in distilled water was added to each sample followed by 3 μL of RNase A (1 mg/mL in water, Sigma Chemical Co., St. Louis, MO, U.S.A.) and the suspension incubated at 37° for 30 min. A 3-μL aliquot of proteinase K (1 mg/mL in water, Boehringer Mannheim, Indianapolis, IN, U.S.A.) was added and the sample incubated for an additional 30 min at 37°. Each sample was then mixed with an appropriate volume of 6× sample loading buffer (0.25% bromophenol blue/40% sucrose in water) and the entire mixture loaded onto an 0.8% agarose gel containing 0.5 μg ethidium bromide/mL. Electrophoresis was performed at 1.5 V/cm of gel length for about 16 hr. DNA bands were visualized using UV transillumination, and photographs of gels were obtained using Polaroid Type 665 positive/negative film.

DNA Fragmentation Assay

DNA fragmentation was assayed as previously described [18]. Briefly, cells (5 × 10^6) were collected by centrifugation (800 *g* for 10 min), lysed with 0.2 mL of ice-cold lysis buffer containing 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, and

* *Abbreviations:* HBSS, Hanks' balanced salt solution; DCFH-DA, 2',7'-dichlorofluorescin diacetate; DCFH, 2',7'-dichlorofluorescin; and DCF, dichlorofluorescein.

0.2% Triton X-100, and pelleted by centrifugation (13,000 *g* for 20 min) to separate intact from fragmented DNA. The supernatant was conserved and the pellet resuspended in 0.2 mL of lysis buffer and sonicated for 10 sec at 4°. DNA concentration in the pellet and supernatant fractions was determined by an automated fluorometric protocol that we designed using AutoAnalyzer II components (Technicon, Tarrytown, NY, U.S.A.) and the DNA-specific fluorochrome Hoechst 33258 (Calbiochem-Behring, La Jolla, CA, U.S.A.). The percentage of DNA fragmentation refers to the ratio of the amount of DNA present in the 13,000 *g* supernatant (fragmented) to the total of the DNA in the pellet (unfragmented) and 13,000 *g* supernatant.

Peroxide Measurements

A sensitive fluorometric method was used to quantitate intracellular peroxides to determine whether ebselen affected peroxide levels in irradiated thymocytes. This method has been used previously to quantitate picomolar concentrations of hydrogen peroxide and lipid hydroperoxides [19, 20]. The assay is based on the fact that the nonpolar, non-fluorescent DCFH-DA can diffuse through the cell membrane and be deacetylated by cytosolic esterases to form the polar, non-fluorescent DCFH. DCFH is trapped within the cytoplasm where it is available to react with peroxides to form the fluorescent compound DCF, a reaction that can be monitored fluorimetrically [21].

DCFH-DA was purchased from Molecular Probes (Junction City, OR, U.S.A.). Stock solutions of DCFH-DA (50 mM) were prepared in DMSO and stored in 100-μL aliquots in the dark at −20°. Thymocytes (1×10^7 cells/mL) were resuspended in Na^+ Hanks' buffer containing 25 μM DCFH-DA and incubated at 37° for 30 min prior to irradiation. Fluorescence measurements were made at room temperature at selected times postirradiation with an SLM 8000 Spectrofluorometer (SLM Instruments Inc., Urbana, IL, U.S.A.) using a stirred cuvette and excitation and emission wavelenghts set at 485 and 530 nm, respectively (band widths 4 nm). Cell viability was not affected by incubation with 25 μM DCFH-DA.

Lipid Peroxidation Measurements

Stock solutions of *cis*-parinaric acid (1 mM) were prepared in 100% ethanol, through which nitrogen had been bubbled for 15 min to remove oxygen. Stocks were stored in 1-mL aliquots in screw-top vials at −20°. For experiments, cells (1×10^7 cells/mL) were incubated in RPMI containing 5 μM *cis*-parinaric acid (Molecular Probes) for 1 hr before irradiation, and then were transferred to Na^+ Hanks' buffer without *cis*-parinaric acid immediately before irradiation. Fluorescence measurements were carried out at room temperature using a stirred cuvette with excitation and emission wavelengths set at 324 and 425 nm, respectively (band widths 4 nm).

RESULTS

The effect of ebselen on morphological changes associated with apoptosis in irradiated (5 Gy, 24 hr post-irradiation) thymocytes is shown in Fig. 1. Panels A and B are bright-field and fluorescence photomicrographs of unirradiated thymocytes, demonstrating the smoothly contoured, similarly sized cells containing evenly distributed DNA that is typical of normal cells. Irradiated thymocytes (Fig. 1, C and D) exhibited morphological changes typical of apoptosis, including cell shrinkage, chromatin condensation, membrane blebbing, nuclear fragmentation, and formation of DNA-containing apoptotic bodies. These morphological changes typically began to appear in thymocytes 16–24 hr after radiation exposure. Incubation of irradiated cells with ebselen for 24 hr beginning immediately after radiation exposure resulted in cells with normal morphology (Fig. 1, E and F), indicating that ebselen completely blocks the morphological changes characteristic of radiation-induced apoptosis. The morphology of unirradiated cells incubated with ebselen for 24 hr was not affected by incubation with the drug (data not shown).

Figure 2 shows the effect of different concentrations of ebselen on radiation-induced internucleosomal DNA fragmentation, a biochemical marker for apoptosis. DNA fragmentation was measured using a sensitive, quantitative fluorometric method developed in our laboratory [18]. Thymocytes were treated with different concentrations of ebselen immediately after irradiation, and DNA fragmentation was measured 8 hr later. As shown, maximal inhibition of DNA fragmentation occurred with ebselen concentrations of about 10 μM and greater.

The effect of ebselen on the viability of unirradiated and irradiated thymocytes was determined by measuring the capacity of these cells to exclude trypan blue. Unirradiated cells incubated with 25 μM ebselen for 8 hr remained 94% viable, a value not different from DMSO-treated (vehicle-control) cells (Table 1). Radiation decreased the viability of otherwise untreated cells to 81%, but irradiated cells incubated with 25 or 50 μM ebselen remained 89 and 86% viable, respectively (Table 1). The results indicate that ebselen is not significantly toxic to cells in this range of concentrations. Based on these results and the effect of ebselen on DNA fragmentation (Fig. 2), we decided to use 25 μM ebselen for the remainder of our studies.

Figure 3 shows the effect of ebselen on the progression of DNA fragmentation in irradiated thymocytes. In these experiments, ebselen was added immediately after radiation exposure and DNA fragmentation was monitored for 8 hr. Fragmentation began 2–4 hr postirradiation (5 Gy) and increased thereafter almost linearly with time. Ebselen treatment not only blocked the radiation-induced fragmentation of DNA but reduced it to levels below the background of fragmentation observed in thymocytes treated with DMSO alone. Figure 4 shows agarose gels of fragmented DNA isolated from thymocytes after various treatments. Gel electrophoresis was used to confirm the pres-

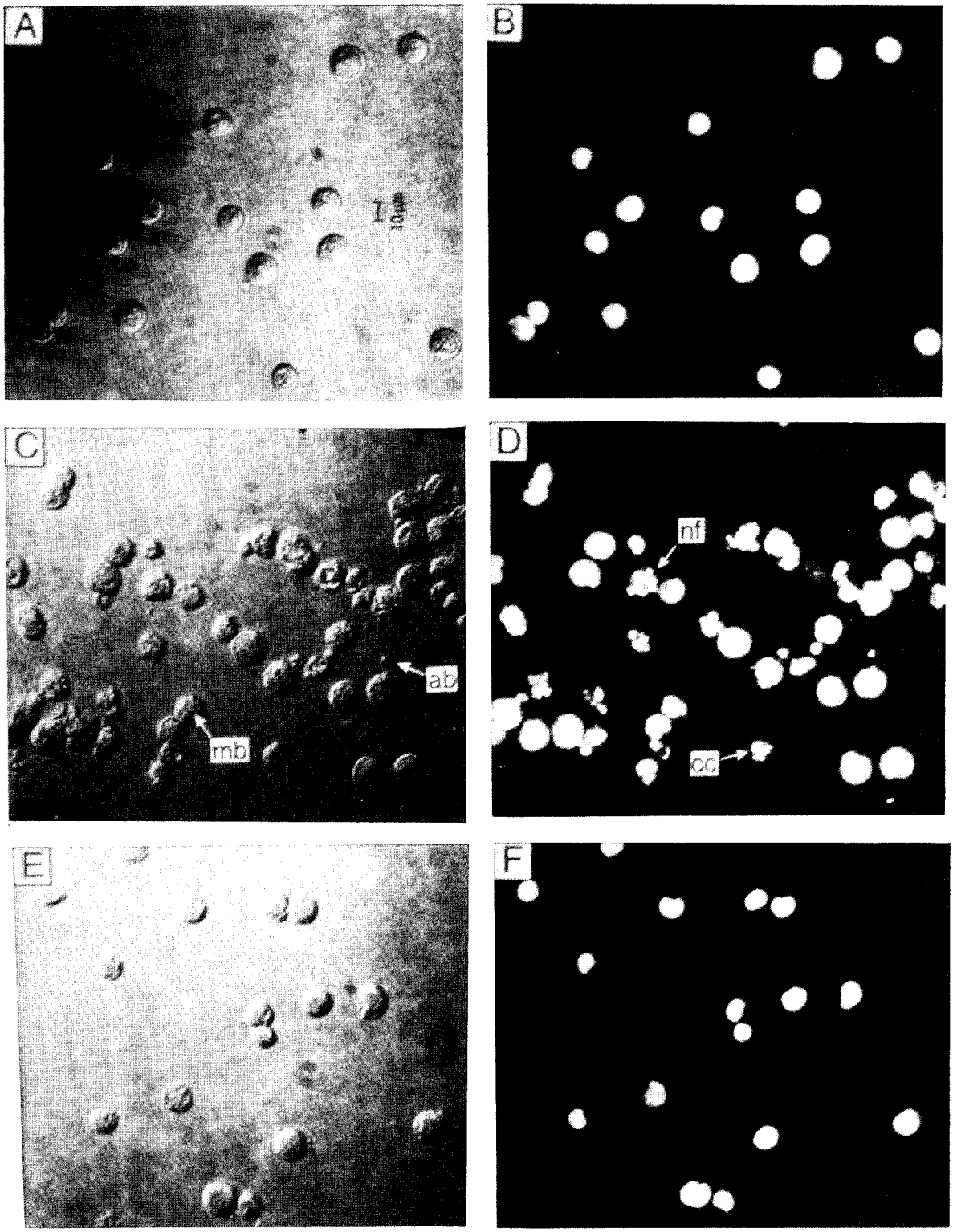

FIG. 1. Effect of ebselen on morphological changes associated with apoptosis in irradiated thymocytes. Thymocytes were prepared for microscopy as described in Materials and Methods. Panels A and B show, respectively, bright-field and DNA-fluorescence images of unirradiated thymocytes, indicating normal morphology. Panels C and D show analogous images 24 hr after radiation exposure (5 Gy, 0.5 Gy/min). Radiation induces morphological changes characteristic of apoptosis, including cell shrinkage, plasma membrane blebbing (mb), and formation of apoptotic bodies (ab). DNA staining shows pronounced chromatin condensation (cc), nuclear fragmentation (nf), and presence of DNA fragments in apoptotic bodies. Panels E and F demonstrate the effect of ebselen on the morphology of irradiated cells. Cells in these images were irradiated and then incubated with 25 μM ebselen for 24 hr postirradiation. Neither bright-field nor fluorescence images exhibited apoptotic morphology. Unirradiated cells treated with ebselen were no different than unirradiated cells not treated with ebselen (data not shown).

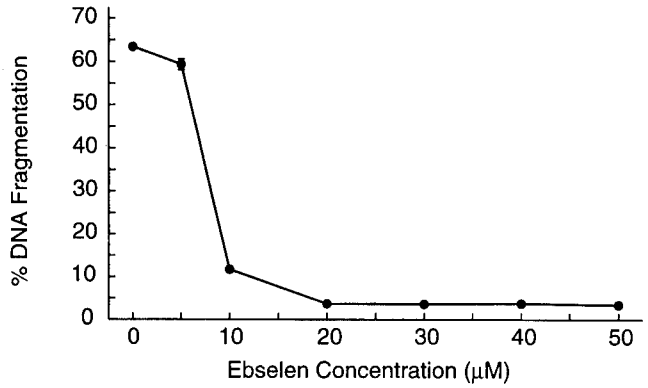

FIG. 2. Influence of different concentrations of ebselen on radiation-induced DNA fragmentation. Thymocytes were incubated with indicated concentrations of ebselen immediately after radiation exposures (5 Gy, 0.5 Gy/min). Fragmented DNA was measured 8 hr after irradiation as described in Materials and Methods. Data represent means ± SEM of 3 experiments.

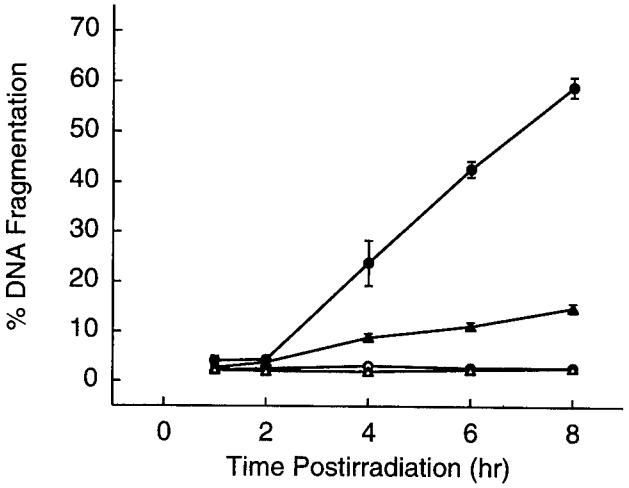

FIG. 3. Effect of ebselen on the time-course of DNA fragmentation in irradiated and unirradiated thymocytes. Unirradiated and irradiated thymocytes were incubated with 25 μM ebselen beginning immediately after irradiation (5 Gy, 0.5 Gy/min). Cell suspensions not treated with ebselen were brought to the same concentration of DMSO equivalent to that delivered with ebselen. Fragmented DNA was measured as described in Materials and Methods at indicated times. Key: (●) 5 Gy; (○) 5 Gy + ebselen; (▲) 0 Gy; and (△) 0 Gy + ebselen. Data represent means ± SEM of three experiments.

ence of apoptotic DNA. The internucleosomal cleavage of DNA during apoptosis produced a "ladder" pattern of DNA fragments on the gel that were multiples of approximately 200 base pairs. There was a small amount of DNA fragmentation detected in unirradiated thymocytes that represented the background fragmentation in untreated cells (lane 2). DNA isolated from irradiated thymocytes showed a typical, pronounced fragmentation pattern (lane 4). Consistent with the data presented in Fig. 3, ebselen completely blocked the appearance of the ladder pattern in both unirradiated and irradiated cells (lanes 3 and 5, respectively). Note that even background fragmentation was no longer apparent.

In all of the studies described above, thymocytes were incubated with ebselen immediately after irradiation. To determine when ebselen treatment of irradiated cells was most effective, thymocytes were treated with ebselen for either 30 min before or during irradiation, after which cells were transferred to medium without ebselen. The results of these experiments (Fig. 5) indicate that ebselen treatment before or during irradiation inhibits DNA fragmentation almost as effectively as treatment after radiation exposure.

A very sensitive fluorimetric method employing DCFH-DA was used to quantitate intracellular peroxides. Incubation of the cells with DCFH-DA began 30 min before irradiation, and the cells were maintained in the presence of the drug throughout the course of the experiments. Fluorescence was then monitored various times after irradiation. Figure 6 summarizes the results of those experiments. Fluorescence intensity in irradiated thymocytes not treated with ebselen increased in a biphasic manner, quickly reaching levels 10–11 times higher than preirradiation levels within the time of the first measurement (2 min postirradiation), followed by a slower rise to 16 times initial levels by 2 hr postirradiation. The early increase in fluorescence intensity after irradiation in ebselen-treated cells was 50% that measured in irradiated cells not treated with ebselen, indicating that ebselen inhibits the formation of radiation-induced peroxides. Ebselen appeared to block completely the second, slower rise in fluorescence that was measured between 2 min and 2 hr postirradiation in irradiated cells not treated with ebselen. Note that there was a "spontaneous" generation of peroxides in unirradiated thymocytes not exposed to ebselen. The level of peroxides in these cells after a 2-hr incubation was about three times that present in the cells before incubation. Our data show that ebselen inhibited this peroxide increase as well.

Peroxides and free radicals produced during irradiation can react with polyunsaturated fatty acids in the membrane to initiate lipid peroxidation reactions. The kinetics of lipid peroxidation during apoptosis in irradiated thymocytes were analyzed using *cis*-parinaric acid, a naturally fluorescent polyunsaturated fatty acid that readily incorporates into cell membranes [22]. Peroxidation of this fatty acid results in loss of fluorescence, which can be monitored over

TABLE 1. Effect of ebselen on the viability of unirradiated and irradiated thymocytes

Incubation conditions	Viable cells (%)	
	O Gy	5 GY
DMSO	95.0 ± 0.8	81.3 ± 0.3
Ebselen (25 μM)	93.6 ± 1.0	88.8 ± 0.1
Ebselen (50 μM)	86.1 ± 1.0	85.6 ± 1.2

Unirradiated and irradiated thymocytes were incubated with indicated concentrations of ebselen until viability was determined 8 hr after irradiation. Cells excluding trypan blue were considered viable. Initial viability was 92.4 ± 0.6%. Data represent means ± SEM of three independent measurements.

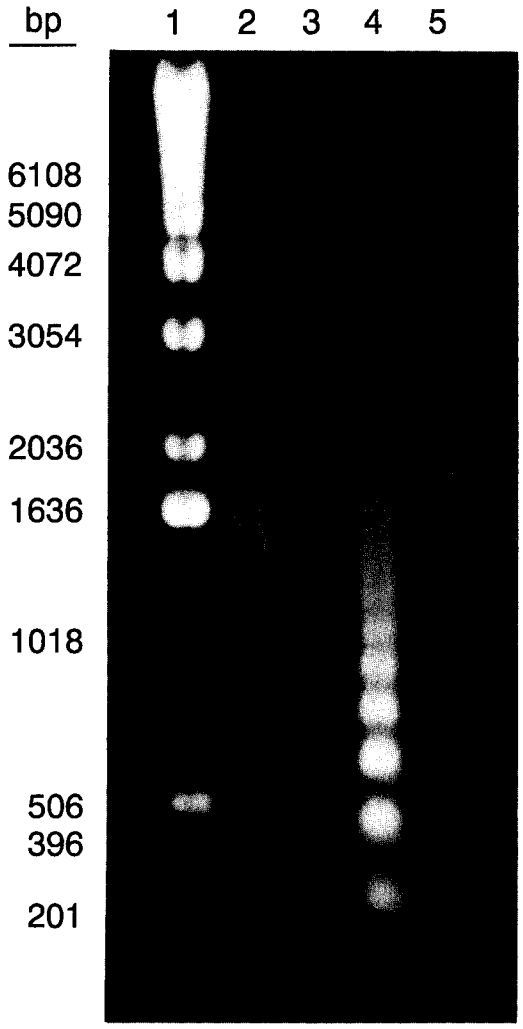

FIG. 4. Effect of ebselen on internucleosomal DNA fragmentation. Fragmented DNA fraction was extracted from irradiated cells (5 Gy, 0.5 Gy/min) and electrophoresed as described in Materials and Methods. Lane 1, standard 1 kb DNA ladder. Lane 2, unirradiated cells after an 8-hr incubation at 37° under 5% CO_2 in air. Lane 3, unirradiated cells incubated with 25 μM ebselen for 8 hr. Lane 4, irradiated thymocytes 8 hr postirradiation. Lane 5, irradiated thymocytes after an 8-hr incubation with 25 μM ebselen.

time [23]. The measurements show (Fig. 7) that the loss of fluorescence began soon after irradiation, with 5% of *cis*-parinaric acid becoming oxidized within 2 min, and the oxidation increased with time. A small amount of oxidation also occurred in unirradiated cells, probably caused by the oxidation of *cis*-parinaric acid by "spontaneous" intracellular peroxides produced as a consequence of incubation conditions, a result consistent with the data in Fig. 6.

DISCUSSION

Apoptosis plays a major role in development, homeostasis, and many diseases including cancer, acquired immune deficiency syndrome, and neurodegenerative disorders [1]. It has been shown that apoptosis induced by a variety of stim-

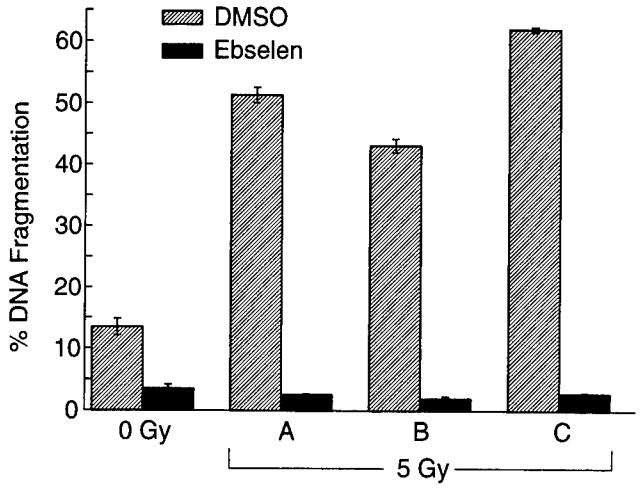

FIG. 5. Optimal timing of ebselen treatment relative to radiation exposure. (A) Preirradiation treatment. Thymocytes were incubated with 25 μM ebselen 30 min prior to irradiation (5 Gy, 0.5 Gy/min). Cells were pelleted and resuspended in fresh medium without ebselen immediately before irradiation. (B) Ebselen during exposure. Ebselen was added to the cell suspension immediately before irradiation. Cells were pelleted and resuspended in fresh medium without ebselen immediately after irradiation. (C) Postirradiation treatment. Cells were incubated with ebselen beginning immediately after irradiation. DNA fragmentation was measured in all samples 8 hr after irradiation. Data represent means ± SEM of three experiments.

uli is associated with increased oxidation levels in the cell. These increases may result from either an overproduction of reactive oxygen species or an attenuation of cellular antioxidant defense systems [12, 24]. The antioxidant defense

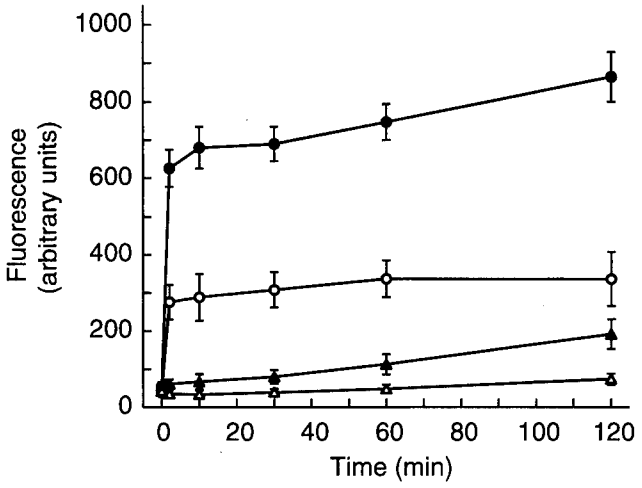

FIG. 6. Peroxide levels in irradiated thymocytes treated or not treated with ebselen prior to radiation exposure. Cell suspensions in Na^+ Hanks' buffer were incubated with 25 μM DCFH-DA at 37° for 30 min prior to irradiation (10 Gy, 0.5 Gy/min) and maintained in the same buffer during measurements. Ebselen-treated cells were exposed continuously to 25 μM ebselen beginning 30 min before irradiation. Key: (●) 10 Gy; (○) 10 Gy + ebselen; (▲) 0 Gy; and (△) 0 Gy + ebselen. Data represent means ± SEM of four experiments.

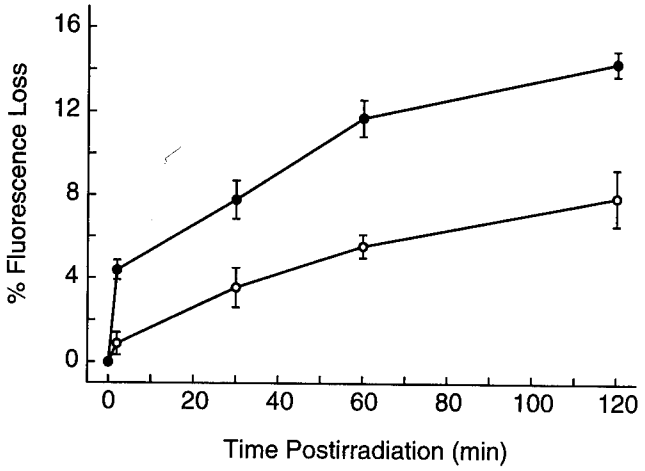

FIG. 7. Lipid peroxidation in irradiated thymocytes. Cell suspensions in medium were incubated with 5 μM *cis*-parinaric acid for 1 hr before irradiation. Cells were resuspended in Na^+ Hanks' buffer without *cis*-parinaric acid prior to irradiation (10 Gy, 0.5 Gy/min) and maintained in the same buffer during measurements. Key: (●) 10 Gy; and (○) 0 Gy. Data represent means ± SEM of three experiments.

system consists of two components: (1) radical scavengers, which quench free radicals, and (2) peroxidases, which prevent radical formation by reducing hydroperoxides to their corresponding, harmless, hydroxy derivatives. There are pharmacological agents that inhibit apoptosis by blocking the production of free radicals, including *N*-acetylcysteine [25], *N*-(2-mercaptoethyl)-1,3-propanediamine [18], and trolox [8, 9, 26]. There have been no previous reports of agents that protect cells from apoptosis by enhancing peroxidase activity. Several studies indicate that the drug ebselen exhibits glutathione peroxidase-like activity similar to that of phospholipid hydroperoxide glutathione peroxidase [15, 27–32]. This report presents results of experiments that are the first to show that ebselen protects cells from radiation-induced apoptosis. These findings also provide circumstantial evidence to support a role for peroxides in signaling apoptosis in the irradiated cell.

The results of these experiments clearly demonstrate that ebselen is an effective inhibitor of the indicators of apoptosis in irradiated thymocytes. First, ebselen treatment preserved normal cell morphology; the plasma membrane appeared normal, chromatin condensation and nuclear fragmentation were absent, and apoptotic bodies were scarce in irradiated cell preparations (Fig. 1). Second, ebselen treatment reduced DNA fragmentation to levels even below those of unirradiated controls (Figs. 3 and 4). Third, the viability of ebselen-treated cells was improved (Table 1).

The inhibition of apoptosis mediated by ebselen, along with the fact that the biological effectiveness of ebselen lies in its capacity to detoxify peroxides, implies that peroxides play a role in radiation-induced apoptosis, but it is not known whether peroxides actually signal (cause) apoptosis. However, our data showing the potent capacity of ebselen to block radiation-induced peroxide generation, combined with the time course of peroxide production in irradiated thymocytes relative to that of apoptosis, suggest that such a causal relationship might exist.

Intracellular peroxides can be detected in thymocytes very early after irradiation—before the morphological and biochemical indicators of apoptosis. A rapid increase in peroxide levels occurred within 2 min postirradiation, leading to levels 16 times the preirradiation values by 2 hr (Fig. 6). This time course of peroxide production preceded DNA fragmentation, which began 2–4 hr after irradiation (Fig. 3), and morphological changes, which became evident 16–24 hr postirradiation (Fig. 1). Ebselen treatment greatly reduced the net production of peroxides in irradiated cells. Unirradiated cells treated with ebselen also generated lower peroxide levels than unirradiated cells not treated, an indication that ebselen reduces even the spontaneous generation of peroxides that occurs during incubation of the cells (Fig. 6).

The mechanism by which radiation-induced peroxides might induce apoptosis remains a mystery, but a variety of observations made by us and others allow us to propose a hypothetical pathway. Evidence seems to indicate that lipid peroxidation is a key step in the process. Lipid peroxidation can be initiated in the irradiated cell by reaction of hydrogen peroxide with polyunsaturated fatty acids in the membrane. Our results indicate that lipid peroxidation chain reactions begin soon after irradiation and increase with time (Fig. 7). These lipid peroxidation reactions appear to play an important role in radiation-induced apoptosis, because the lipid peroxyl radical scavenger trolox effectively inhibits apoptosis in thymocytes and human MOLT-4 cells induced by either ionizing radiation [8, 9] or hydrogen peroxide [26]. Other reports have also implicated lipid peroxidation as playing a role in apoptosis [12, 25, 33]. Oxidized lipids can subsequently be acted upon by phospholipases, which can lead to the release of fatty acid hydroperoxides from the membrane. It is known that lipid hydroperoxides can induce apoptosis when added to cells, an activity that may be related to their capacity to increase cytoplasmic calcium [34]. Little is known of the link between elevated cytoplasmic calcium and apoptosis, but a number of studies have shown that an increase in cytosolic calcium occurs during apoptosis [8, 9, 35], an event that may trigger signal transduction pathways.

The observation that ebselen was effective if added postirradiation is consistent with the proposal that lipid hydroperoxides may be key intermediates in radiation-induced apoptosis. They are formed predominantly postirradiation (as indicated by the time course of membrane lipid peroxidation), so ebselen need be present only after exposure to exert its effect. Ebselen, however, also inhibited apoptosis when cells were incubated with the drug only before and during irradiation (followed by transfer of the cells to ebselen-free medium). Such results do not necessarily diminish the importance of the postirradiation reactions. It is possible that effective concentrations of ebselen may persist in the cell even after a short incubation if ebselen quickly enters and is retained in the cytosolic and

membrane compartments. Even small amounts of ebselen may provide effective antioxidant activity, because ebselen behaves as a catalyst and as such is not consumed during detoxification reactions [15].

In our experiments, we measured significant levels of DNA fragmentation not only in irradiated but also in unirradiated cells, though at much lower levels [approximately 60 vs 15% fragmentation, respectively, at 8 hr postirradiation (Fig. 3)]. This "spontaneous" DNA fragmentation was also correlated with the increases in peroxides (Fig. 6) and lipid peroxidation (Fig. 7) in untreated cells. We believe these events are a consequence of *in vitro* incubation conditions. After thymocytes were removed from the animal, the cells were incubated in standard cell culture incubators in an atmosphere containing just under 20% oxygen, a much higher pO_2 level than *in vivo*. This relatively high O_2 level could lead to oxidative stress in the cells. The fact that ebselen decreased both peroxide production and DNA fragmentation (apoptosis) in unirradiated cells probably results from the capacity of ebselen to block oxidative damage that occurs in these cells as a result of the incubation conditions.

Ebselen is not toxic to thymocytes under the conditions of our experiments; viability of unirradiated cells treated with ebselen was not appreciably different than that of untreated cells (Table 1). *In vivo* studies with human volunteers and animals also indicate that ebselen has no toxicity [29, 36, 37]. The absence of selenium toxicity probably results from the fact that selenium is covalently bound to an organic matrix from which it is not released [31, 36, 37]. Its biochemical activity and its low toxicity have led to its therapeutic use in humans. Ebselen is now in Phase I clinical trials as an antiinflammatory drug [38], based on its glutathione peroxidase-like action in scavenging peroxides produced during various pathological inflammatory conditions [14, 39]. It may prove rewarding to explore the use of ebselen as a therapeutic agent for protecting cells from apoptosis induced by peroxides released during development [12, 40], acquired immune deficiency syndrome [12, 24], radiation exposure, and other pathological conditions [1].

To summarize, our experiments indicated that ebselen is an effective inhibitor of radiation-induced apoptosis in thymocytes. Ebselen was not significantly toxic to the cells and could be added before, during, or after irradiation to exert its effect. The data suggest that ebselen exerts its effect by detoxifying primary and/or secondary peroxides produced during and after irradiation. The results also provide new insights into the general role of lipid peroxidation in radiation-induced apoptosis. Ebeselen or drugs with similar activity may prove to be useful tools in treating diseases associated with the inappropriate triggering of apoptosis.

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Comparison of the Effects of Typical and Atypical Anxiolytics on Learning in Monkeys and Rats[1]

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ABSTRACT

Atypical anxiolytics such as buspirone have been reported to produce fewer disruptive effects on complex behaviors than some typical anxiolytics from the benzodiazepine class. To extend this analysis, several drugs from both drug classes were directly compared in two species (rhesus monkeys and rats) using a repeated-acquisition procedure. In monkeys responding under a multiple schedule of reinforcement consisting of acquisition (learning) and performance components, buspirone (0.032–0.56 mg/kg), 8-hydroxy-dipropylaminotetralin (8-OH-DPAT;0.032–0.56 mg/kg), chlordiazepoxide (CDZP; 1–56 mg/kg) and alprazolam (0.032–0.32 mg/kg) produced dose-dependent decreases in overall response rate in all subjects. However, with buspirone and 8-OH-DPAT, these rate-decreasing effects occurred in acquisition at lower doses than in performance. The effects on overall accuracy (*i.e.*, percent errors) in monkeys were variable across drugs and drug classes. Both 8-OH-DPAT and alprazolam produced large increases in percent errors in acquisition at doses that had little or no effect on errors in performance. Buspirone also had differential effects on percent errors across components, but the error-increasing effects in acquisition were smaller. CDZP administered either orally or intramuscularly produced only small increases in errors, and these occurred at doses that substantially decreased the overall rate of responding in both components of the multiple schedule. In rats responding under a repeated-acquisition procedure, buspirone (1–5.6 mg/kg), 8-OH-DPAT (0.056–3.2 mg/kg) and CDZP (1.8–32 mg/kg) produced dose-dependent decreases in overall response rate. Similar to acquisition data in monkeys, buspirone and 8-OH-DPAT also increased percent errors to a greater extent than CDZP. These data indicate that learning is sensitive to disruption by drugs with 5-HT_{1A} agonist properties, and that atypical anxiolytics with 5-HT_{1A} agonist properties are no less disruptive to "cognitive" processes than typical anxiolytics such as the benzodiazepine alprazolam.

Clinical trials have demonstrated that the partial 5-HT_{1A} agonist buspirone (Buspar) is effective in the treatment of anxiety, with efficacy and dosage comparable to benzodiazepines such as diazepam (Taylor *et al.*, 1985; Pecknold *et al.*, 1989). The apparent absence of anticonvulsant, sedative and muscle-relaxant effects with buspirone has been thought to be a substantial advantage over other anxiolytics, and allows buspirone to be characterized putatively as "anxioselective." This characterization resulted from studies that reported that buspirone had a decreased interaction with alcohol and a decreased level of psychomotor impairment compared with anxiolytic benzodiazepines. In fact, as Taylor (1988) states in a review of the clinical properties of buspirone, it is preferred with anxiety states that present with cognitive and interpersonal problems, including anger and hostility, whereas diazepam is preferred for anxiety states that present with muscle tension and insomnia due to its well-known muscle-relaxant properties.

Few studies, however, have directly compared 5-HT_{1A} agonists and the benzodiazepines for the ability to disrupt complex behavioral procedures requiring acquisition or learning (*e.g.*, Panickar and McNaughton, 1992). More important, this comparison has rarely been made while also testing subjects on a performance task. The following experiment tested two 5-HT_{1A} agonists with differing selectivities for the $5HT_{1A}$ receptor (*i.e.*, buspirone and 8-OH-DPAT) and two benzodiazepines with differing structures (CDZP and alprazolam) in monkeys responding on a complex behavioral base line involving both learning and performance. As an additional means of testing the assertions concerning the atypical anxiolytics, three of these four drugs (buspirone, 8-OH-DPAT and CDZP) were also administered to rats whose behavioral base line also consisted of a learning task. Alprazolam (Xanax) was administered to monkeys because it is a tria-

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine (serotonin); 8-OH-DPAT, 8-hydroxy-dipropylaminotetralin; CDZP, chlordiazepoxide.

zolobenzodiazepine approved for the treatment of anxiety and panic disorders in humans. In the treatment of anxiety, alprazolam has been shown to be as efficacious as the longer acting 1,4-benzodiazepine diazepam in both animal studies (File and Pellow, 1985; Söderpalm *et al.*, 1989) and human studies (Aden and Thein, 1980; Greenblatt *et al.*, 1993). As indicated by Aden and Thein (1980), one advantage of alprazolam over diazepam in humans may be that there tends to be fewer reported side effects such as drowsiness and lightheadedness at anxiolytic doses.

The procedures used to test both monkeys and rats in this experiment involved a repeated-acquisition technique. Briefly, these procedures required that a subject learn a different predetermined sequence of responses (of fixed length) each session to obtain reinforcement. In this case, reinforcement for both species of experimental animal consisted of food presentations under a second-order fixed-ratio schedule. As Cole (1986) points out in his review of the effects of benzodiazepines on acquisition and performance, appetitive paradigms are particularly well suited for investigating the effects of benzodiazepines because decrements might safely be attributed to learning or memory impairment, not to motivational changes associated with the appetite-enhancing properties of the benzodiazepines. The same cannot be said for aversive paradigms in which shock is characteristically used, because there is likely to be significant confounding of the anxiolytic and memory-altering effects of benzodiazepines in such contexts. An additional advantage of this base line is that during a given session, learning is demonstrated by the behavioral pattern of responding where the subject makes fewer incorrect responses and a greater number of consecutive correct responses as the session progresses. Across sessions, this type of learning is repeatedly tested as the predetermined sequence is changed with each new session. As an additional control for the nonspecific effects of each drug, a performance component was included in the base line for the monkeys. Similar multiple schedules have been used to test the effects of anxiolytics in monkeys (Moerschbaecher *et al.*, 1983; Thompson and Moerschbaecher, 1979) and humans (Bickel *et al.*, 1990; Desjardins *et al.*, 1982).

Methods

Subjects

Five domestic born adult male rhesus monkeys, *Macaca mulatta*, served as subjects. Monkeys were housed in individual stainless-steel cages and food deprived to approximately 95% of their free-feeding weight throughout the experiment. This moderate level of deprivation (5%) was sufficient to produce stable day to day responding, and helped reduce the risk of adventitious health problems. The mean weight for these animals was 10.2 ± 2 kg. Each animal's diet consisted of banana-flavored food pellets (Bio-Serv Inc., Frenchtown, NJ), monkey chow, fresh fruit and vitamins. Water was available *ad libitum* in the home cage. For behavioral testing, each subject was removed from the colony-room cage and transported via a macaque restrainer (Primate Products, Inc., Redwood City, CA) to experimental chambers located in another room.

Six male Sprague-Dawley rats also served as subjects in this experiment. All rats were maintained at 80% of their free-feeding weight (mean 372.2 ± 32 g) by food presented during the experimental session and by supplemental postsession feeding. Subjects were housed individually in plastic Micro-Isolator cages (Allentown Caging Equipment Co., Inc., Allentown, NJ) containing sterilized hardwood-chip bedding. Similar to the monkeys, water was available *ad libitum* in the home cage, and each subject was tested in an operant chamber.

The colony rooms for both monkeys and rats were provided 10 air changes/hr of 100% fresh air, conditioned to 21 ± 1°C with 50 ± 10% relative humidity and were maintained on a 12 L: 12 D cycle (no twilight), which began at 6:00 A.M. each day. At the start of the experiment, all five monkeys were drug naive except for those occasions when animals were sedated for periodic tuberculosis testing and physical examinations. The rats were also drug naive when the experiment began. All research was conducted according to the principles enunciated in the Guide for the Care and Use of Laboratory Animals, prepared by the Institute of Laboratory Animal Resources, National Research Council.

Apparatus

Monkeys. Each monkey was tested in a sound-attenuating experimental chamber with an operant panel located on the front wall. The inside dimensions of the chambers were 136 cm × 93 cm × 72 cm, which permitted the monkeys seated in the restrainers to be positioned at approximately arms-length from the panel. Each aluminum panel (measuring 54 cm × 35.5 cm) contained three translucent response keys aligned horizontally (8 cm apart, center to center at eye level) and two recessed response levers, which were located on both sides of the pellet trough approximately 15.5 cm below the response keys. An in-line stimulus projector, mounted behind each key, projected colors and/or geometric forms onto the key. In addition to these stimuli, a single white jewel lamp was located directly over each response lever, and a third white lamp was located 7.5 cm above the middle response key. Response keys required a minimum force of 0.15 N for activation, and each correct response on a key produced an audible click of a feedback relay. Each chamber was illuminated with overhead incandescent lights located near the back of the chamber. A fan mounted on the top of each chamber provided continuous ventilation. During experimental sessions, white noise was present in the test room to mask extraneous sounds.

Rats. Four identical modular test chambers (Coulbourn Instruments, Inc., model E10–10TC, Allentown, PA) configured specifically for rodents were used. The front wall of each chamber contained a houselight, speaker, auditory feedback relay, pellet trough (10 cm above the floor and centered), and three response keys aligned horizontally (8 cm apart, center to center and 4.5 cm above the floor). Each response key could be transilluminated by three Sylvannia 28ESB indicator lamps, one with a red plastic cap, one with a green cap and one with a yellow cap. Response keys required a minimum force of 0.15 N for activation, and correct responses produced an audible click of the feedback relay. Each chamber was enclosed within a sound-attenuating cubicle equipped with a fan for ventilation. White noise was continuously present in each chamber to mask extraneous sounds.

Both monkey and rat test chambers were connected to a PDP11/73 computer (Digital Equipment Corp., Bedford, MA) programmed in SKED-11 software (State Systems, Inc., Kalamazoo, MI) and to cumulative recorders (Gerbrands Corp., Arlington, MA) located in a nearby room.

Procedure

Monkeys. A multiple schedule with acquisition and performance components served as the base line. During the acquisition component, all three response keys were illuminated at the same time with one of four colors, yellow, green, red or white. The monkey's task was to respond (key press) on the correct key in the presence of each sequentially illuminated set of colors (*e.g.*, keys yellow–center correct; keys green–left correct; keys red–center correct; keys white–right correct). When the chain was completed, the keylights turned

off, and the stimulus lamp over the right lever was illuminated. A press on this lever extinguished the lamp over the lever, produced a 0.4-sec presentation of the pellet trough light and reset the chain. The same sequence (in this case, center-left-center-right or CLCR) was repeated throughout a given session and was maintained by food presentation under an FR 5 schedule; *i.e.*, every fifth completion of the sequence produced a 500-mg food pellet when the food lever was depressed. When the subject pressed an incorrect key (in the example, the left or right key when the white keylights were presented), the error was followed by a 5-sec timeout. During timeouts, keylights were turned off and responses had no programed consequence. An error did not reset the four-response sequence; *i.e.*, the stimuli were the same before and after the timeout.

To establish a steady state of repeated acquisition, the four-response sequence was changed from session to session. An example of a typical set of six sequences was LRCR, CLRL, LRLC, RCRL, CLCR, RCLC, with the order of the color presentations always yellow, green, red and white. The sequences were carefully selected to be equivalent in several ways, and there were restrictions on their ordering across sessions (see Thompson, 1973). Briefly, each of the 17 sequences were scheduled with equal frequency and adjacent positions within a sequence for a given session were different.

During the performance component of the multiple schedule, a small triangle was projected onto the center of all three keys simultaneously with the color that identified each link in the four-response sequence. Unlike the acquisition component, the four-response sequence in this component remained the same from session to session (*i.e.*, LCLR). In all other aspects (FR 5 schedule of food presentation, timeout duration of 5 sec, etc.), the performance component was identical to the acquisition component.

Each session for monkeys began in the acquisition component, which then alternated with the performance component after 10 reinforcements or 15 min, whichever occurred first. Sessions for monkeys terminated after 100 reinforcements or 2 hr, whichever occurred first.

Rats. The repeated-acquisition procedure for rats was simplified in that the subjects were only required to learn a three-response sequence each session, and there was no performance component. A typical set of five sequences was CLR, RCL, LRC, CRL and RLC, with the order of the color presentations always green, red and yellow. Unlike the monkey procedure, a single correct sequence position for a given color was occasionally the same for two consecutive sessions (as in the above list of sequences, LRC and CRL), and the sequence ended after the third correct key press (*i.e.*, no additional food-lever response was required to illuminate the pellet trough and reset the sequence). Additionally, food presentations occurred under an FR 2 schedule; every second completion of the sequence illuminated the pellet trough (0.4 sec) and produced a 45-mg food pellet. Similar to the monkey procedure, errors produced a brief 5-sec timeout during which all stimuli were turned off. Sessions for rats were terminated after 200 reinforcements or 90 min, whichever occurred first.

Drugs

The drugs used were buspirone hydrochloride, chlordiazepoxide hydrochloride, alprazolam (all from Sigma Chemical Co., St. Louis, MO) and (±)-8-hydroxy-2-(di-n-propylamino)tetralin hydrobromide (8-OH-DPAT, Research Biochemicals, Inc., Natick, MA). Doses of buspirone and chlordiazepoxide were dissolved in sterile saline (0.9%). Alprazolam was dissolved in a vehicle of 0.9% saline (60%) and propylene glycol (40%). The injection volume for i.m. administration of all the drugs in monkeys was 0.05 ml/kg body weight. At higher doses of CDZP, solubility at the concentration of 0.05 mg/ml was a problem, so the ratio of saline to drug was increased, and multiple injections were given. When CDZP was administered orally to monkeys, the drug was mixed at a concentration of 0.1 mg/ml, and then 0.1 ml/kg body weight was added to 20 ml of vehicle (fruit punch). For rats, the injection volume for both saline and drug was always 0.1 ml/100 *g* body weight. For the monkeys, presession times for drug and control injections were 15 min for buspirone and 8-OH-DPAT, 60 min for alprazolam and 180 min for CDZP. Oral administration of CDZP occurred 90 min before the start of the session. For rats, the presession time for the three drugs was 15 min. For both monkeys and rats, the doses of each drug were tested in a semirandom order. At least 2 wk of base-line sessions intervened between the end of a series of injections with one drug and the start of a series with another. Drug sessions were generally conducted on Tuesdays and Fridays, with control sessions conducted on Thursdays. Higher doses of all the drugs, particularly of CDZP, were administered only once a week.

Data Analysis

The data for each session were analyzed in terms of 1) the overall response rate (total responses/min, excluding timeouts) and 2) the overall accuracy, expressed as percent errors [(errors/total responses) × 100]. The data for each subject were analyzed by comparing drug sessions with control (saline or vehicle) sessions. Drug dosages were considered to have an effect to the extent that the mean data for a given dosage fell outside the ranges of variability established during control sessions for that drug. In addition to these measures based on session totals, within-session changes in responding were monitored by a cumulative recorder and a computer. For example, acquisition of a response sequence was indicated by within-session error reduction, *i.e.*, a decrease in the number of errors between food presentations as the session progressed.

Results

Stable responding by all five monkeys occurred under both components of the multiple-schedule base line. Furthermore, measures of both rate and accuracy for each subject remained stable during base-line sessions and control sessions when either vehicle or saline was administered. Although overall response rates were generally higher in performance (*e.g.*, see control data for response rates in fig. 1), response rates in both components were consistent from session to session. Accuracy, as indicated by percent errors for each subject, was also stable across sessions in both components. Moreover, the response pattern in the acquisition components in particular was characterized by a steady state in terms of within-session error reduction. Acquisition, which usually occurred a short time after the start of the session (5 min), was characterized by a distinct decrease in the number of errors and an increase in correct completions of the response sequence. This response pattern at the start of the session in acquisition also accounted for the fact that percent errors in acquisition were typically larger than percent errors in performance under control conditions.

When 0.032 to 0.56 mg/kg of buspirone were administered, dose-dependent decreases in overall response rate occurred in both schedule components in all monkeys. As shown in figure 1, these rate-decreasing effects occurred in acquisition at lower doses than they occurred in performance. Differential effects under the two schedule components were also apparent in the accuracy data where buspirone reliably produced small increases in percent errors in acquisition, but little or no increases in percent errors in performance. Although the magnitude of these error-increasing effects was small for each subject, increases in percent errors were evident at several doses.

The effects of 0.01 to 0.56 mg/kg of 8-OH-DPAT on both response rate and percent errors are shown in figure 2. Re-

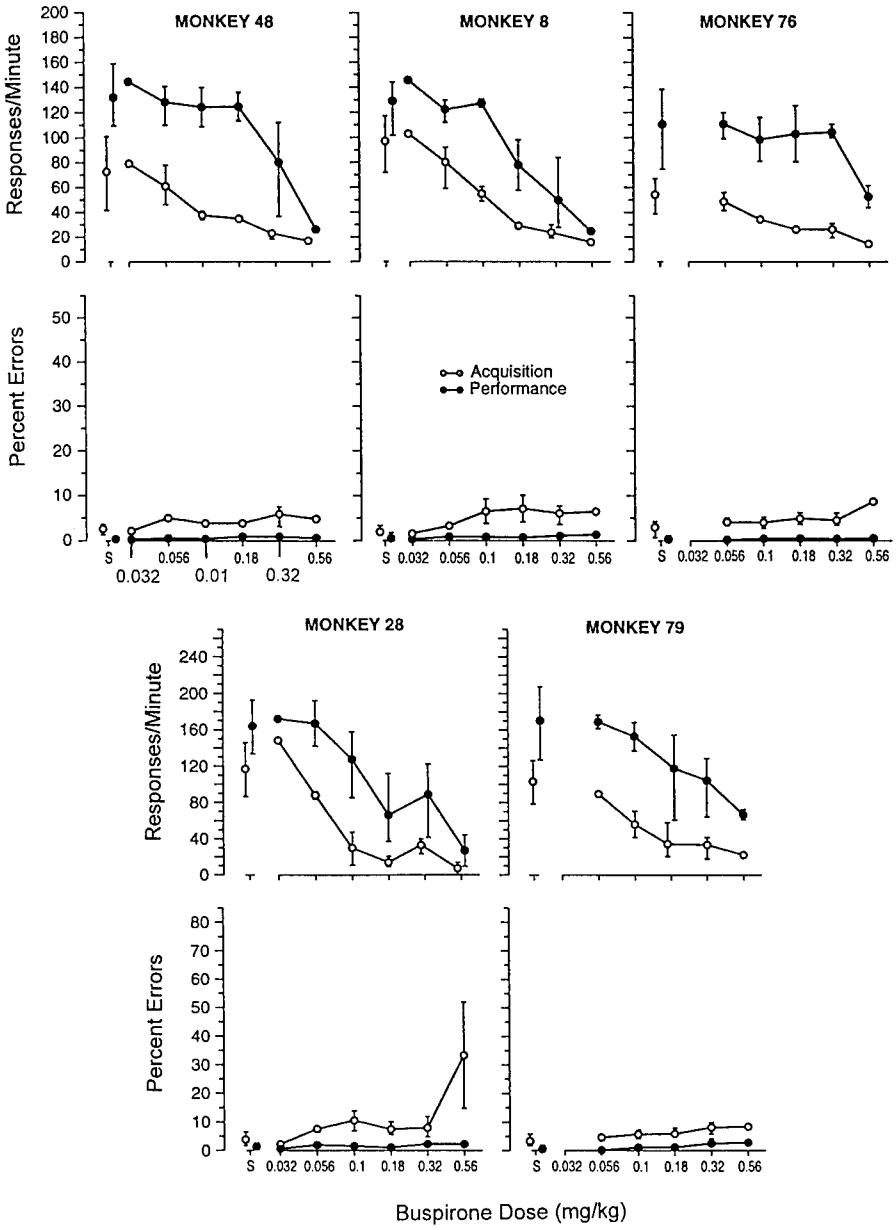

Fig. 1. Effects of buspirone on the overall response rates and percent errors in the acquisition and performance components of the multiple schedule for all five monkeys. The unconnected points with vertical lines to the left of the dose-effect curves at S indicate the mean and range of 14 to 15 saline control sessions for that subject. Any points at S without vertical lines indicate an instance in which the range is encompassed by the data point. The data points with vertical lines in the dose-effect curves indicate the mean and range for two to three determinations of that dose; data points without vertical lines in the curves indicate either a single determination of that dosage or an instance in which the range is encompassed by the data point.

garding the effects on overall response rates, 8-OH-DPAT produced dose-dependent rate-decreasing effects in both components in all five subjects. Similar to buspirone, these rate-decreasing effects generally occurred in acquisition before they occurred in performance. This differential effect on response rate was most evident in the data for monkey 28 where doses from 0.032 to 0.32 produced marked decreases in response rate in acquisition, but no decreases in rate in performance. 8-OH-DPAT was also similar to buspirone in that it produced selective effects on percent errors; *i.e.*, 8-OH-DPAT produced increases in acquisition errors when little or no increases in performance errors were apparent. However, with 8-OH-DPAT, the magnitude of the disruptive effect in acquisition was substantially larger than that found with buspirone. For example, in all the monkeys, except monkey 48, error-increasing effects in acquisition were obtained at doses as low as 0.056 mg/kg and increased to levels well above controls, whereas only small error-increasing effects were obtained in performance and then only at the higher doses.

The highly selective effects produced by 8-OH-DPAT are also shown in figure 3 by the within-session patterns of responding for monkey 8. Each cumulative record in this figure shows the effects produced during an entire session. As shown in the early portion of the control record (top row), and more closely by the first inset, errors in acquisition generally decreased to near zero levels shortly after the session began. This characteristic decrease in errors and increase in errorless responding as the session progressed generally indicated the point at which the subject was considered to have acquired the correct sequence of responses. This typical pattern of errors in acquisition was in direct contrast to the pattern of errors in performance, which consistently reflected an error rate near zero at the start of the session and one that remained near zero throughout the control session.

In this subject, 0.032 and 0.056 mg/kg of 8-OH-DPAT pro-

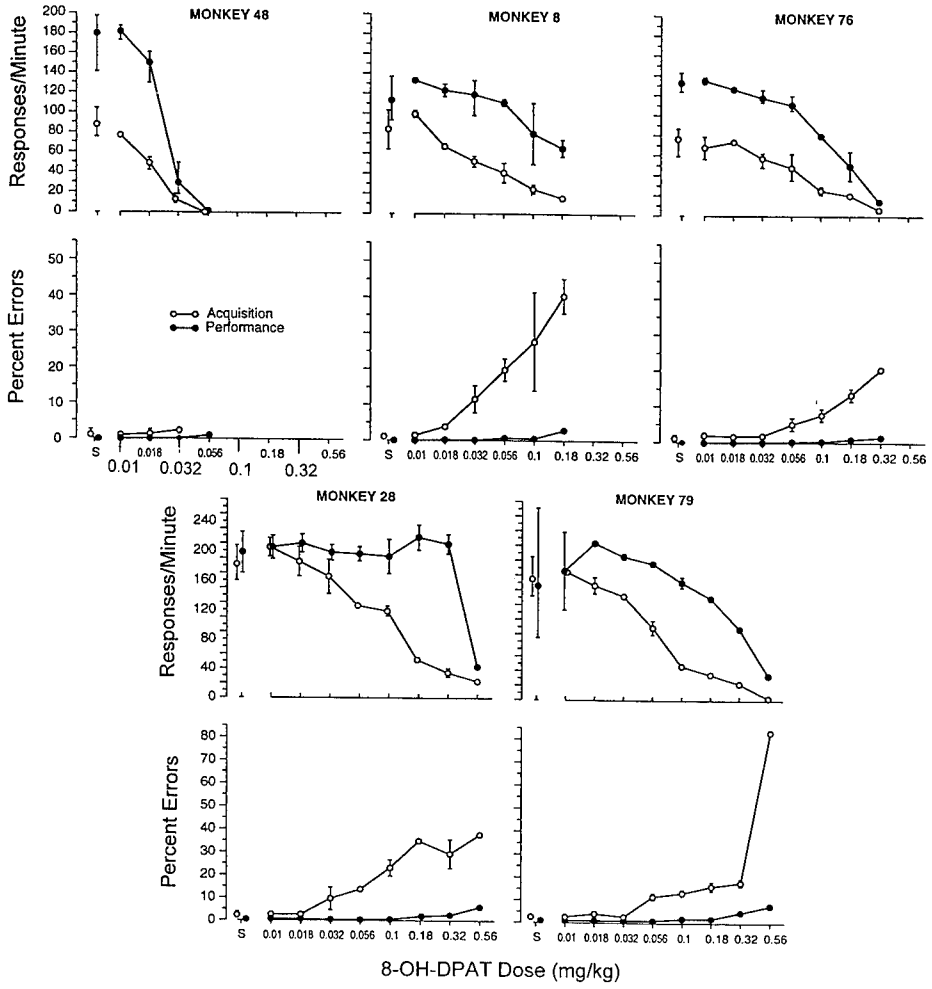

Fig. 2. Effects of 8-OH-DPAT on the overall response rates and percent errors in the acquisition and performance components of the multiple schedule for all five monkeys. Data points at S indicate the mean and range of 16 to 18 saline control sessions. For additional details, see legend for figure 1.

duced a pattern of errors in acquisition that was distinctly different from the control pattern even though the overall rate of responding was not decreased substantially. In the record for both doses, this effect was represented by an increase in the number of errors (lower pen) in each of the acquisition components for the sessions. The differential effects across schedule components are highlighted in the second inset, which represents the effects of 0.056 mg/kg of 8-OH-DPAT in the initial acquisition and performance components of that session. As shown, this dose produced a marked increase in errors and a decrease in consecutive errorless completions of the response sequence in acquisition, but little or no effect in performance.

After the administration of the 0.1 and 0.18 mg/kg doses of 8-OH-DPAT, disruptions in accuracy and overall response rate in acquisition were more substantial than those seen at intermediate doses, and disruptions in the performance component also occurred. However, the effects in acquisition remained proportionally greater in acquisition than performance. For example, in the record for the 0.18-mg/kg dose, large rate-decreasing and error-increasing effects were obtained in acquisition throughout the entire 2-hr session, but the effects obtained in performance were limited to the initial cycles of the performance component.

Figures 4 and 5 show the effects of i.m. and oral CDZP, respectively, in all five monkeys. As shown in both figures, 1 to 56 mg/kg of CDZP by either route of administration produced dose-dependent decreases in overall response rates in both components although producing little or no effects on percent errors in either component. In contrast to buspirone or 8-OH-DPAT, i.m. and oral CDZP at a given dosage had comparable effects on response rates in both schedule components and had only marginal effects on percent errors in both components despite clear decreases in response rates across subjects. One exception is the i.m. CDZP data for monkey 48, which shows comparatively large increases from control levels in percent errors at the 10- and 18-mg/kg doses.

The effects that occurred on both response rates and percent errors 24 hr after i.m. and oral CDZP are also depicted in figures 4 and 5, respectively. In general, the 24-hr effects obtained with both routes of administration were markedly similar to the effects obtained on the day CDZP was administered, indicating a long time course for the behavioral effects of CDZP. With few exceptions, CDZP after 24 hr produced dose-dependent decreases in response rates in both components that were nonselective and increases in percent errors in both components that were comparatively small when present.

Figure 6 depicts the effects of 0.032 to 0.32 mg/kg of alprazolam on both measures of responding in all five monkeys. As shown, this range of doses of alprazolam administered i.m. produced rate-decreasing and error-increasing effects that were dose dependent. In general, the rate-decreasing effects produced with alprazolam were very similar to those pro-

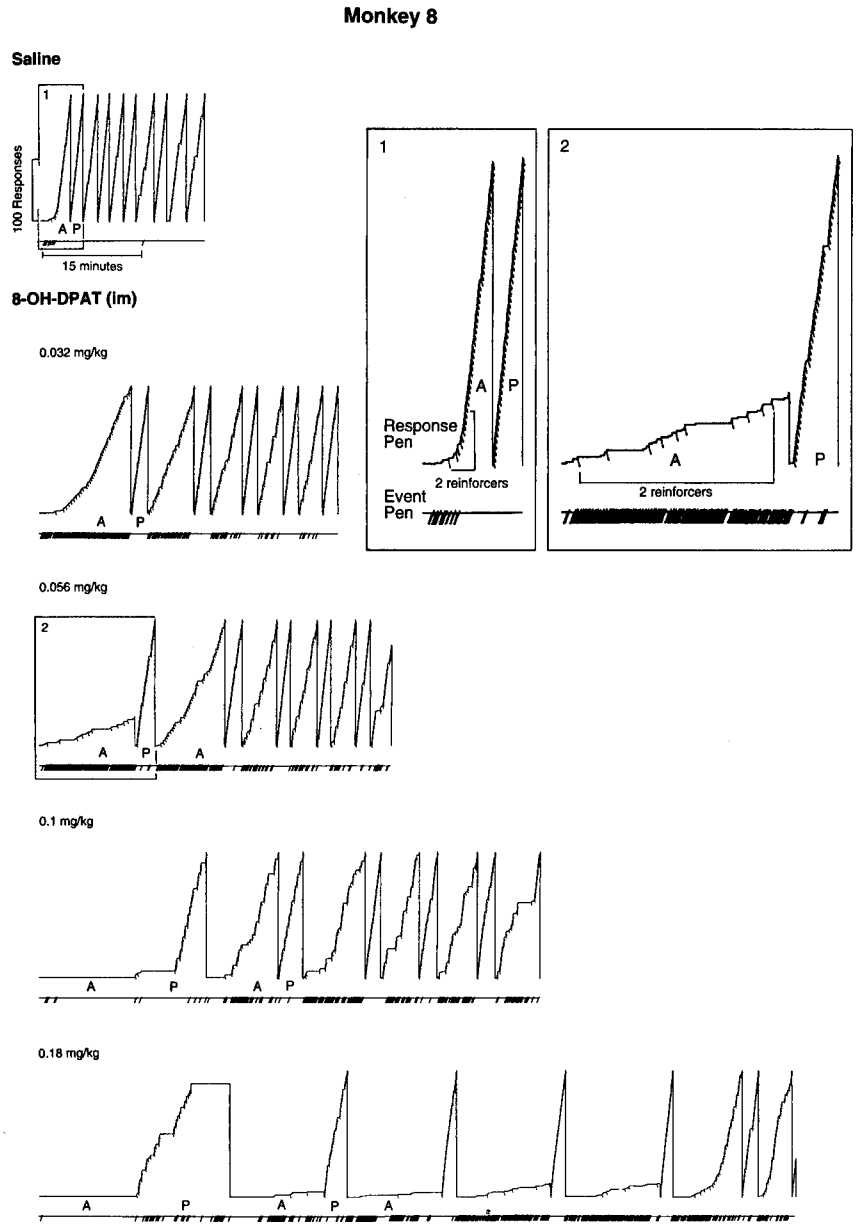

Fig. 3. Cumulative response records for monkey 8 showing the within-session effects produced in the acquisition (A) and performance (P) components of the multiple schedule by 8-OH-DPAT; each record depicts an entire session. As exemplified by the magnified insets, the response pen stepped upward with each correct response and deflected downward each time the four-response sequence was completed. Errors in both components are indicated by the event pen (below each record). A change in components of the multiple schedule, which occurred after 15 min or 100 reinforcements, reset the stepping pen.

duced in individual monkeys by CDZP (see figs. 4 through 6). Furthermore, dose-effect curves for response rates in individual monkeys with i.m. alprazolam were most similar to those found with i.m. CDZP; *i.e.*, an extremely steep dose-effect curve for response rate was obtained with monkey 48, and extremely shallow dose-effect curves were obtained for monkeys 76, 28 and 79.

This same similarity between i.m. alprazolam and i.m. CDZP was, however, not as evident in the accuracy data. In three of the five monkeys, for example, alprazolam produced rather large disruptive effects that were not found with i.m. or oral CDZP (*i.e.*, alprazolam produced rather substantial and selective increases in percent errors in monkeys 8, 28 and 79). In fact, the effects on percent errors in these particular monkeys were larger in magnitude than those found with buspirone or CDZP (i.m. and oral) (see figs. 1, 4, 5 and 6). As with CDZP, alprazolam in monkeys 76 and 48 produced little or no error-increasing effects even though response rates in both schedule components were clearly decreased below control rates of responding.

The cumulative records in figure 7 display some of the differences in within-session effects of each of the drugs when administered to monkey 8. Each record in this figure depicts the effects of a dosage of each drug that decreased the overall response rate in both components of the multiple schedule. The three records in the top row show control sessions in which saline or vehicle (oral and i.m.) were administered. Note that in these records the pattern of responding in both components is comparable after the first acquisition component. In addition, note that responding in the performance components was always characterized by error levels near zero and by high rates of errorless responding.

As indicated by the records in rows 2 to 4, the within-session patterns of responding were altered when each drug was administered before the start of the session, For example, 0.18 mg/kg of buspirone (row 2) eliminated responding

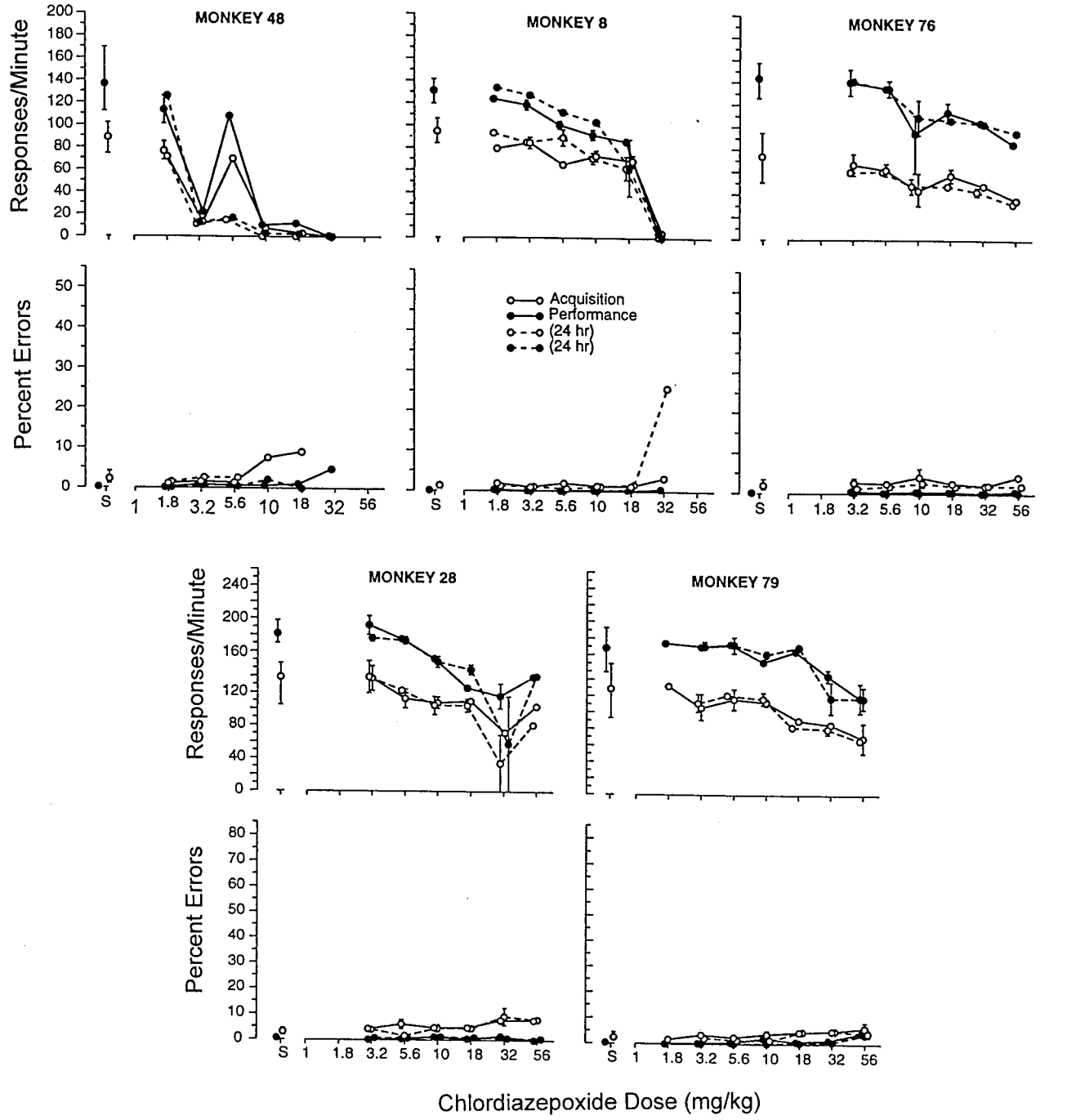

Fig. 4. Effects of i.m. doses of CDZP on overall response rates and percent errors in the acquisition and performance components of the multiple schedule for all five monkeys. Data points at S indicate the mean and range of 10 to 13 saline control sessions. For additional details, see legend for figure 1.

during the entire first acquisition component and a large part of the second acquisition component. Thereafter, responding in the acquisition components was characterized by a substantial increase in errors (compared to control levels) and some additional brief periods of pausing. Also shown in this record, buspirone produced comparatively little effect on performance at this dose (*i.e.*, there was only a small decrease in response rate and almost no effect on errors). Highly selective effects on the within-session pattern of responding can be seen in the record for 0.1 mg/kg of 8-OH-DPAT (row 4). In this record, however, the error-increasing and rate-decreasing effects were present for an extended period. This effect on acquisition was very different from the effect on performance where rate and accuracy were relatively unaffected.

In contrast to these differential effects, the effects of CDZP (administered i.m. or orally) produced little or no effect on accuracy and relatively uniform decreases in response rate in both components. This uniform effect across components also occurred with 0.18 mg/kg of alprazolam, which produced decreases in response rate that were similar in magnitude in both components and comparable to those seen with CDZP. However, alprazolam produced a noticeable increase in errors in each of the acquisition components of the session.

In rats responding under a repeated-acquisition procedure,

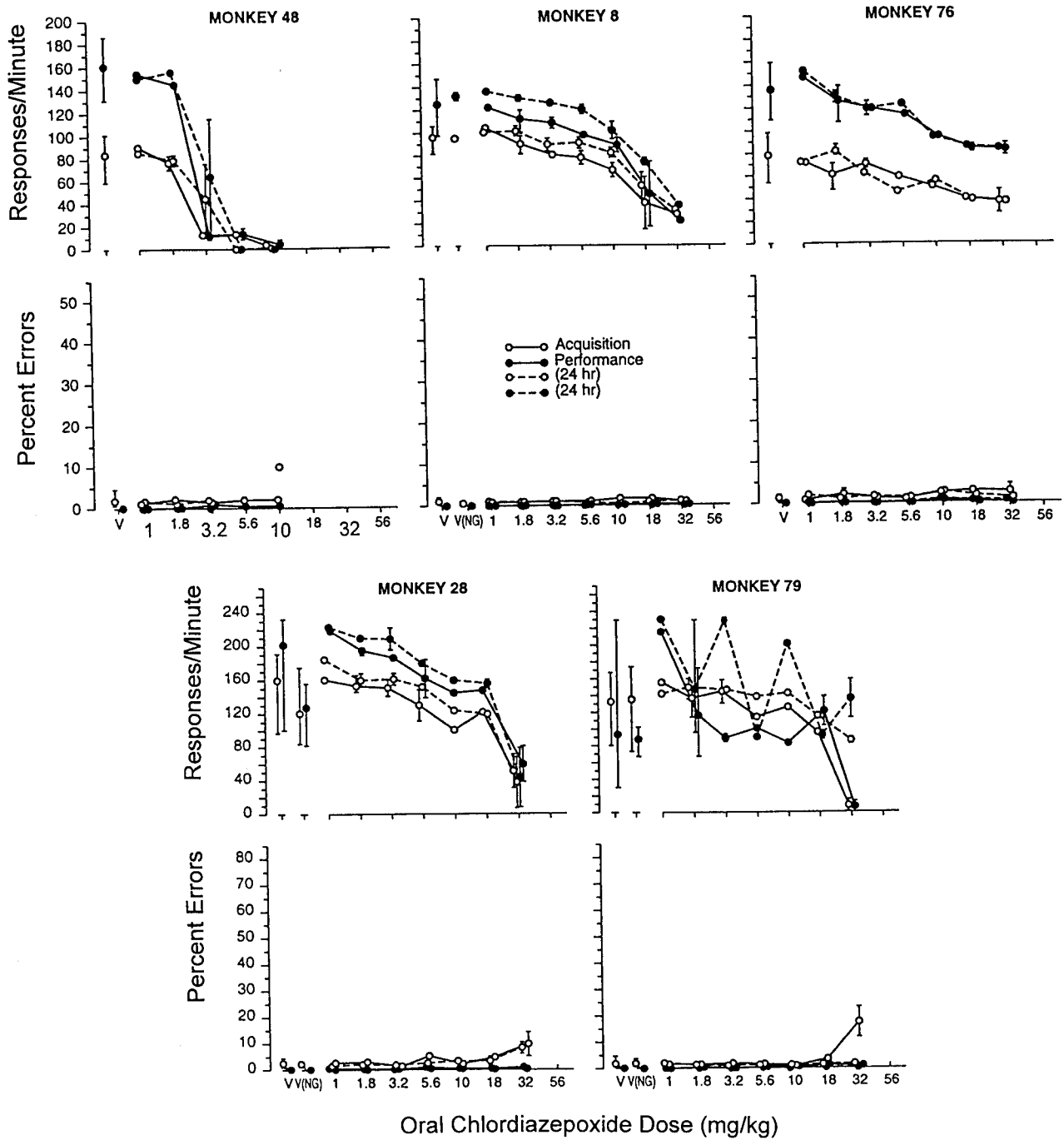

Fig. 5. Effects of orally administered doses of CDZP on overall response rates and percent errors in the acquisition and performance components of the multiple schedule for all five monkeys. Data points at V indicate the mean and range of 19 to 24 vehicle control sessions, whereas points at V(NG) indicate several sessions in which the vehicle was administered by a nasogastric tube. In these particular monkeys, the highest doses of CDZP were also administered by a nasogastric tube due to problems with palatibility of the drug solutions. For additional details, see legend for figure 1.

1 to 5.6 mg/kg of buspirone i.p. produced dose-dependent decreases in overall response rates and increases in percent errors. These effects, which are shown in figure 8, show that buspirone frequently increased errors at doses that had little or no effect on overall response rate. In at least four of six subjects, for example, this effect can be seen at the 3.2- mg/kg dose. Also note that buspirone produced small but reliable increases in overall response rate in RP-13 and RP-14.

When administered either i.p. or s.c. to rats, 8-OH-DPAT (0.056–3.2 mg/kg) dose-dependently decreased overall response rate and increased percent errors in all subjects (fig. 9). Although many lower doses in this dose range did produce similar effects on both rate and accuracy, s.c. administration tended to be more disruptive than i.p. administration. These slightly greater effects of s.c. 8-OH-DPAT can be seen in almost all subjects at the higher doses where the data for s.c. administration are frequently shifted a ¼ log-unit to the left. Additionally, s.c. administration (more often than i.p. administration) produced small rate-increasing effects in some subjects at several lower doses (*e.g.*, RP-11, RP-12 and RP-13)

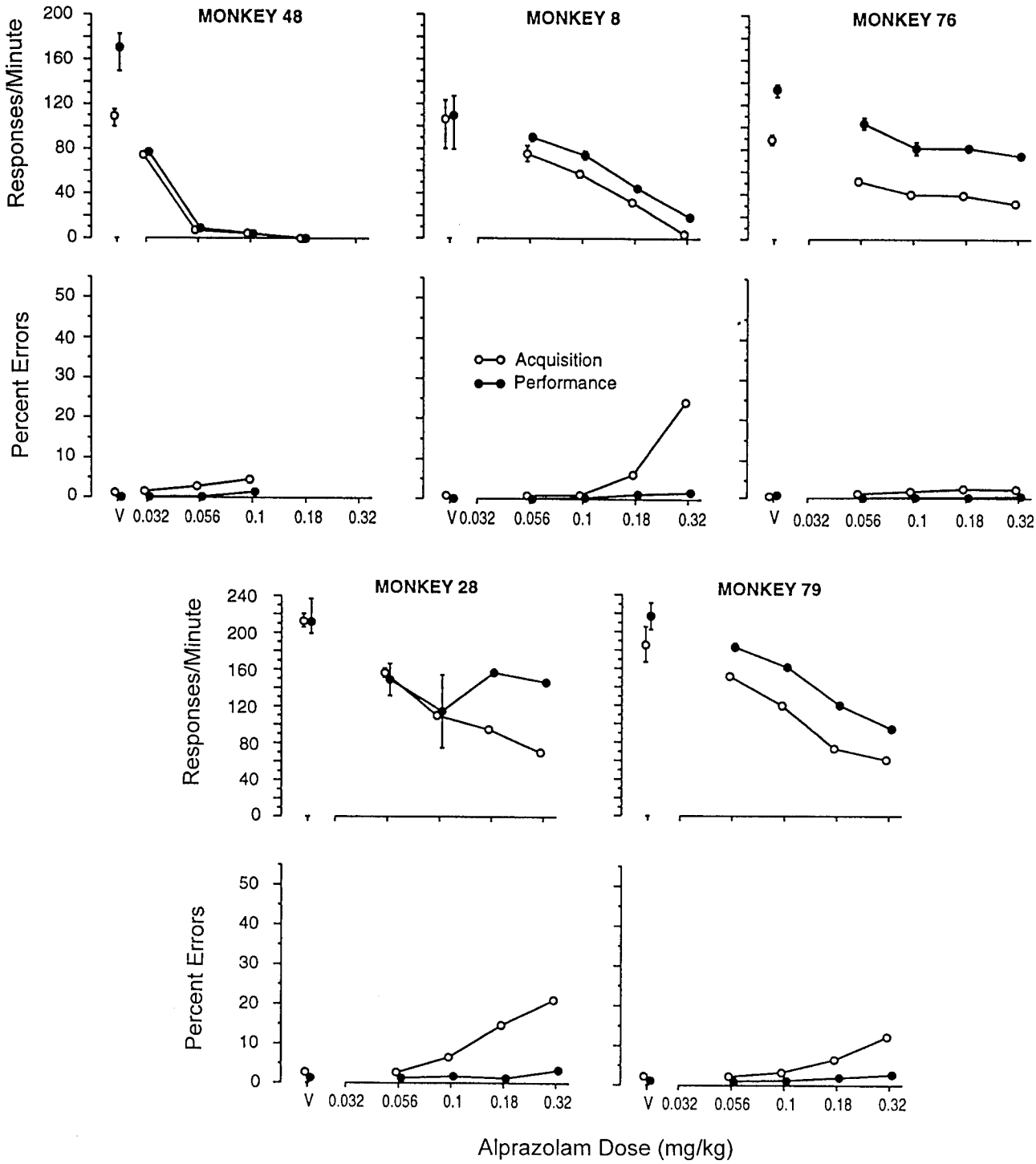

Fig. 6. Effects of alprazolam on overall response rates and percent errors in the acquisition and performance components of the multiple schedule for all five monkeys. Data points at V indicate the mean and range of 5 vehicle control sessions. For additional details, see legend for figure 1.

and produced error-increasing effects when little or no rate-decreasing effects were obtained. This selective effect on percent errors can be seen in five of six rats at the 0.32-mg/kg dose. In RP-13, in particular, large rate-increasing effects were evident after s.c. administration of most of the lower doses.

The effects of 1.8 to 32 mg/kg of CDZP are shown for all rats in figure 10. Lower doses of CDZP produced small rate-increasing effects in four of six rats, whereas higher doses produced rate-decreasing effects in all subjects. CDZP in all rats produced dose-dependent increases in percent errors. Note, however, that the error-increasing effects with CDZP generally occurred at doses that substantially decreased the overall rate of responding. The only marked exception to this finding was the data for the 18-mg/kg dose in RP-9.

Cumulative records in figure 11 show some of the differ-

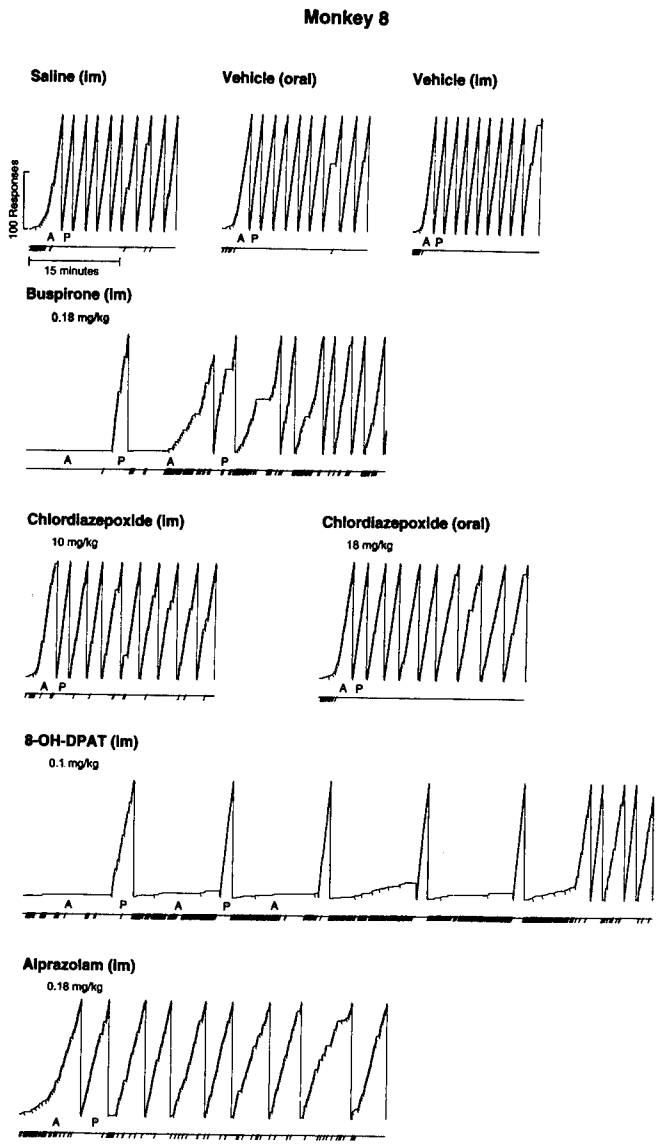

Fig. 7. Cumulative response records showing the within-session effects of a dosage of each drug administered to monkey 8. Each record, including the three control records presented across the top row and the two records for CDZP in the third row, shows the data from an entire session. All sessions terminated after 2 hr or 100 reinforcements, whichever occurred first. For additional details, see legend for figure 3.

ences in within-session effects of each of the drugs administered to rat RP-11. In figure 11, each record shows the effects of a dosage of each drug that produced comparable rates of responding during acquisition. The control record at the top shows the characteristic pattern of responding for this subject when saline was administered i.p. before the start of the session. As can be seen, the subject's error rate decreased within 15 min of the start of the session and the pattern of responding after that time was characterized by long runs of correct sequence completions and only sporadic errors. An arrow above this record indicates a relative transition point in acquisition; *i.e.*, a point where errors began to decrease and consecutive errorless completions of the response sequence increase.

As shown in the records for all three drugs, the within-session patterns of acquisition were disrupted by each of the dosages shown. Moreover, each of the drugs disrupted acquisition somewhat differently despite the comparable rates of responding obtained after each dose. Buspirone administered i.p. (1.8 mg/kg), for example, tended to produce slightly larger increases in errors and decreases in overall response rate at this dose than either i.p. CDZP or i.p. 8-OH-DPAT. Moreover, errors were evident for a longer period of time, and the transition point occurred much later in the session as indicated by the arrow. Also, note that even though the transition points for acquisition occurred at about the same time for i.p. CDZP and for i.p. 8-OH-DPAT (see arrows in rows 3 and 4), i.p. 8-OH-DPAT produced more pausing at the start of the session. Of particular note were the effects of s.c. 8-OH-DPAT (row 5). When administered by this route, 8-OH-DPAT produced greater effects on response rate and produced an error rate that was very high throughout the session such that there was no easily distinguishable transition point indicative of acquisition.

Discussion

The multiple-schedule base line of repeated acquisition and performance in monkeys produced stable rates of responding in both components and produced error levels that were generally higher in acquisition than in performance. This stability across components for both overall rates and errors was critical for repeatedly evaluating the effects of all the drugs tested and was characteristic of the base-line behavior in other repeated-acquisition studies involving both monkeys (Moerschbaecher *et al.*, 1983; Thompson and Moerschbaecher, 1979) and humans (Bickel *et al.*, 1990; Desjardins *et al.*, 1982). In those studies as well as our study, the higher error levels in acquisition under control conditions generally reflected the repeated assessment of learning as the sequence was changed for each daily session. That the variability in error levels for learning fell within a relatively narrow range for each subject reflected the fact that each subject had reached a steady state of transition states (cf. Thompson and Moerschbaecher, 1978), which indicated that essentially the same learning curve was generated with each new sequence a monkey acquired. Another advantage of the multiple schedule was that the pattern of behavior in acquisition could be directly compared with the pattern of behavior in performance where the error rate remained relatively unchanged or near zero throughout the session. Therefore, within each session and throughout this study, responding in performance served as a control for nonspecific effects of each of the drugs tested.

When a similar range of doses of buspirone and 8-OH-DPAT were administered to monkeys before the start of the session, dose-dependent rate-decreasing effects occurred in both components of the multiple schedule. More important, in acquisition, these rate-decreasing effects occurred at lower doses, indicating that behavior in acquisition tended to be more sensitive to these drugs' rate-disrupting effects than behavior in performance. To our knowledge, this finding has not been reported previously for the 5-HT_{1A} receptor agonists in experiments involving either rhesus monkeys or any other species of old world monkey. However, the greater sensitivity of learning has been reported under similar experimental conditions for drugs from other pharmacological classes, such as the benzodiazepines (*e.g.*, Bickel *et al.*, 1990; Thompson, 1975), barbiturates (*e.g.*, Moerschbaecher and Thompson,

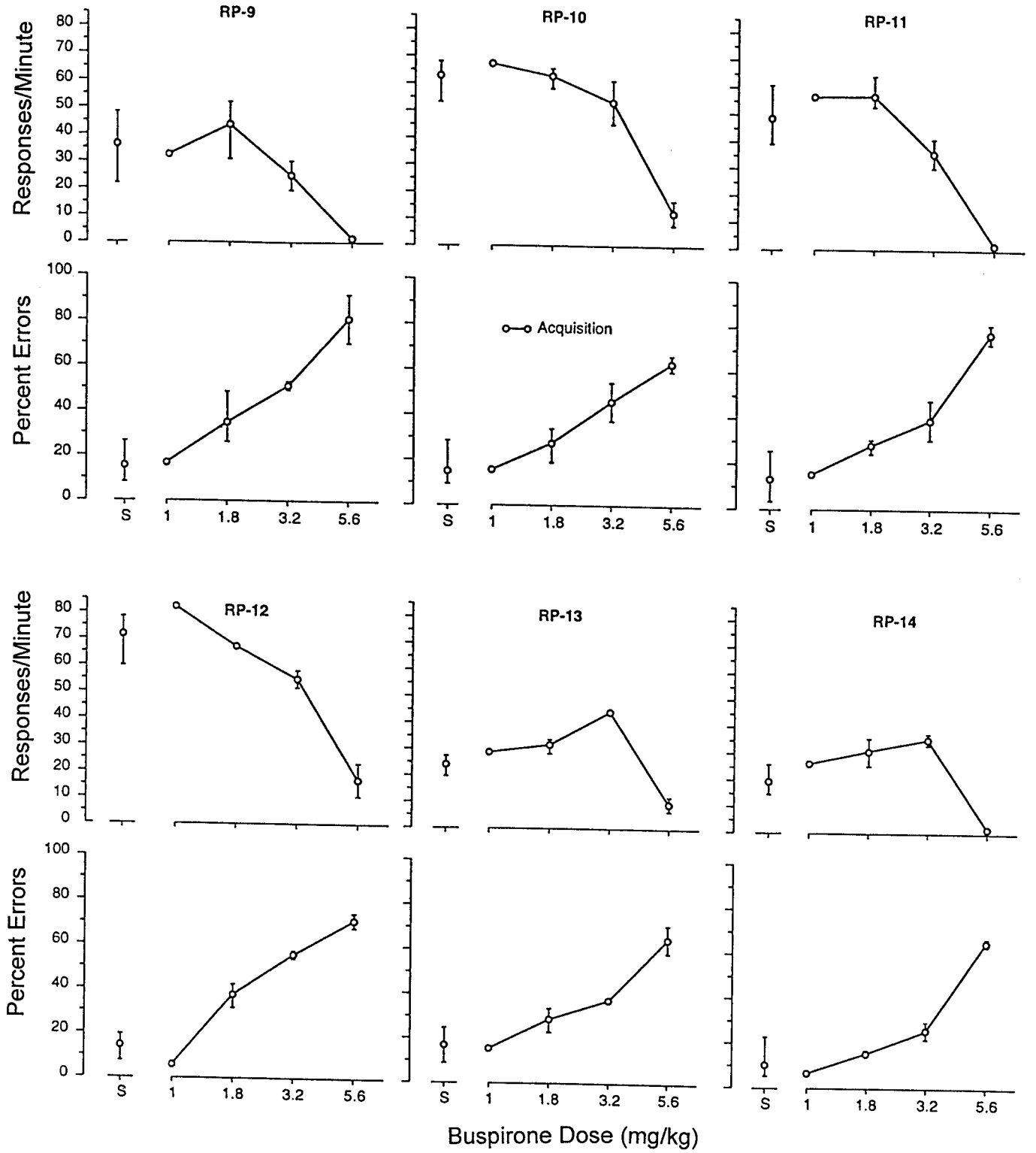

Fig. 8. Effects of buspirone on the overall response rate and percent errors in all six rat subjects responding under a repeated acquisition procedure. In this procedure, all rats were required to learn a different three-response sequence each session, but they were not required to respond under a performance schedule. The unconnected points with vertical lines to the left of the dose-effect curves at S indicate the mean and range of 10 saline control sessions for that subject. Any points at S without vertical lines indicate an instance in which the range in encompassed by the data point. The data points with vertical lines in the dose-effect curves indicate the mean and range for two to three determinations of that dose; data points without vertical lines in the curves indicate either a single determination of that dosage or an instance in which the range is encompassed by the data point.

1980a; Thompson, 1975) and NMDA receptor antagonists (France *et al.*, 1991; Moerschbaecher and Thompson, 1980b; Thompson and Moerschbaecher, 1984). In general, behavioral variables such as task complexity and/or stimulus control (*i.e.*, differential responding in the presence of different stimuli) have been shown to play an important role in the

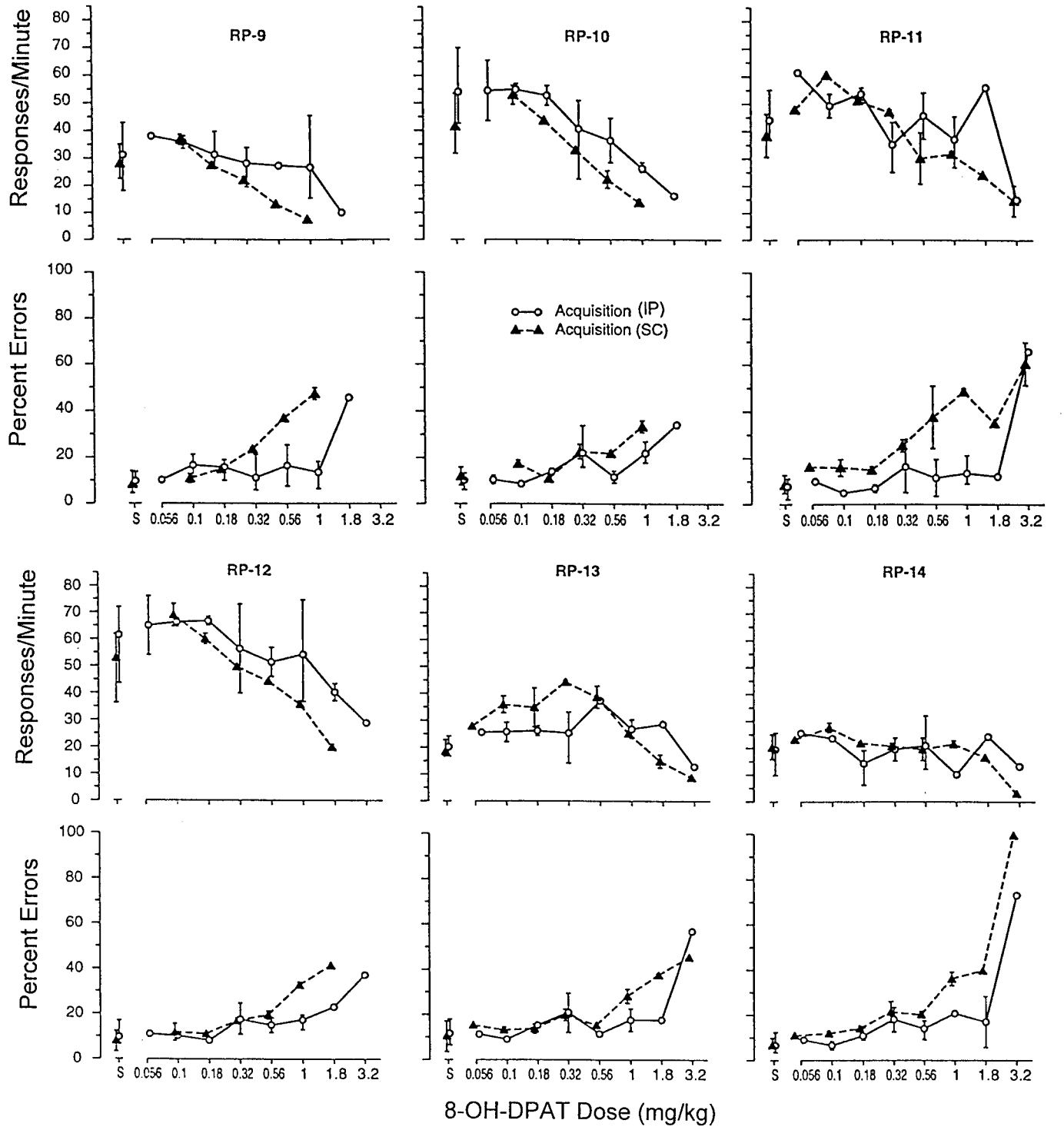

Fig. 9. Effects of 8-OH-DPAT on overall response rate and percent errors in rats responding under a repeated-acquisition procedure. Data points at S indicate the mean and range of 11 to 15 control sessions when saline was administered i.p. and 9 to 13 control sessions when saline was administered s.c. For additional details, see legend for figure 8.

degree to which different behaviors are susceptible to disruption by various drugs (*e.g.*, Laties *et al.*, 1981; Thompson, 1975; Thompson and Moerschbaecher, 1979; Winsauer *et al.*, 1985). Certainly, in our experiment, the behavior in acquisition was under weaker stimulus control because the stimuli for correct responding changed in this component with each daily session.

Across a substantially higher range of doses (3–30 mg/kg), buspirone has been shown to produce rate-decreasing effects in squirrel (new world) monkeys responding under a multiple schedule with both unpunished and punished components (Weissman *et al.*, 1984). Under this multiple schedule, responding in both components was maintained under a FR-30 schedule, and only the 30-mg/kg dose decreased the overall rate of responding in the unpunished (food-maintained) component. This finding in monkeys supported the general ob-

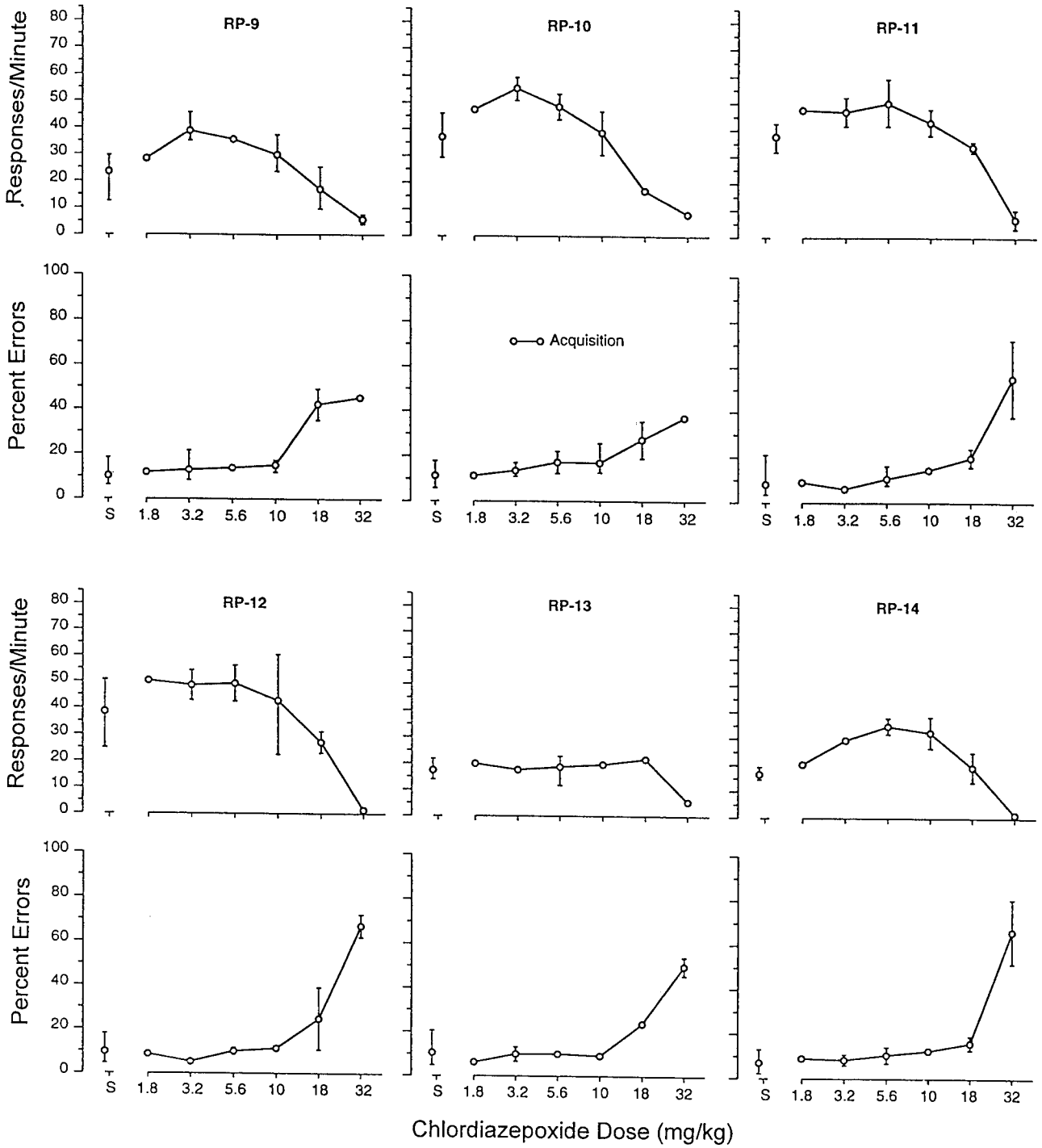

Fig. 10. Effects of CDZP on overall response rate and percent errors in rats responding under a repeated-acquisition procedure. Data points at S indicate the mean and range of 16 to 20 saline control sessions. For additional details, see legend for figure 8.

servation that the rate-altering effects of buspirone on punished responding occur at doses lower than those required to disrupt unpunished responding. Given buspirone's reported anxiolytic effects in humans (Pecknold *et al.*, 1989; Taylor, 1985) and animals (File and Andrews, 1994), most of the behavioral research involving animals and operant procedures has examined the effects of the 5-HT_{1A} receptor agonists on conflict responding or responding that is suppressed with shock (Gleeson and Barrett, 1990; Mansbach *et al.*, 1988; McCloskey *et al.*, 1987; Weissman *et al.*, 1984; Wettstein, 1988; Witkin and Barrett, 1986; Witkin and Perez, 1990). Other studies have looked at the 5-HT_{1A} receptor agonists on active or passive avoidance tasks (*e.g.*, Geller and Hartmann, 1982; Rowan *et al.*, 1990; Sanger *et al.*, 1989). As in the studies mentioned above, Geller and Hartmann (1982) found that a relatively high dose range of buspirone (0.5–7.5 mg/kg) was required to disrupt avoidance responding.

Of particular note in our study were the effects of the 5-HT_{1A} receptor agonists on the accuracy of responding. Two

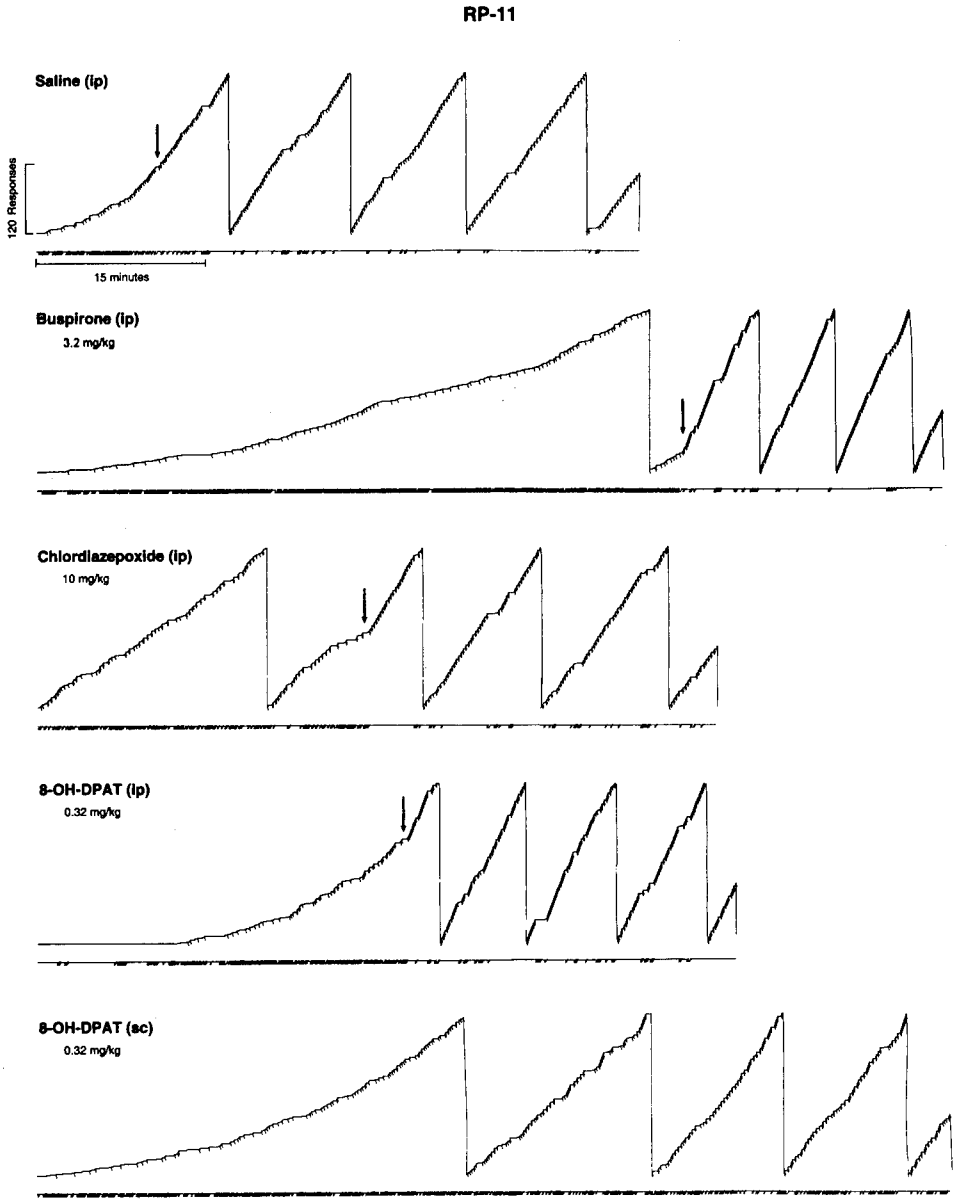

Fig. 11. Cumulative response records showing the within-session effects of a dosage of each drug administered to rat RP-11. Each record depicts an entire session. All sessions for rats terminated after 90 min or 200 reinforcements, whichever occurred first. The arrows indicate relative transition points in the within-session pattern of responding or acquisition of the response sequence. For additional details, see legend for figure 3.

important findings were 1) the apparent selectivity of these agonists for disrupting learning and 2) the rather substantial difference in the ability of buspirone and 8-OH-DPAT to selectively produce disruptive effects on learning even though both are considered 5-HT_{1A} receptor agonists. In addition, data from drug discrimination studies involving both pigeons and rats have shown that these two compounds cross-generalize to one another (*e.g.*, Cunningham *et al.*, 1987; Nader *et al.*, 1989; Sanger and Schoemaker, 1992). To date, more research has been conducted on animals performing some sort of complex learning or memory task with buspirone than with 8-OH-DPAT, and most of these reports, which involved a large variety of procedures and species of experimental animal, have indicated that buspirone can be disruptive (Bass *et al.*, 1992; McNaughton and Morris, 1992; Rowan *et al.*, 1990). For example, Rowan *et al.* (1990) found that both retention of a passive avoidance task and acquisition of a Morris water-maze task were disrupted by doses of buspirone in the 1- to 2-mg/kg range. In contrast, little comparative information currently exists on the ability of 8-OH-DPAT to disrupt different types of learning procedures.

Similarly to our study, many of the human studies involving buspirone have compared the effects of buspirone with those of a specific benzodiazepine. Unlike the data in our study, which indicated that buspirone was more disruptive than CDZP, most of these studies have reported that buspirone is much less likely to cause disruptions across a range of procedures (for review see Lister, 1991). In one study, for example, 20 mg of buspirone administered for 9 consecutive days was found to be less disruptive to driving-related skills than 15 mg of diazepam given over the same period of time (Moskowitz and Smiley, 1982). These investigators also reported that chronic buspirone administration actually improved performance, whereas chronic diazepam administration disrupted performance. This overall difference in effect reported for humans and monkeys when buspirone is compared with the benzodiazepines remains difficult to explain. Certainly, the accumulation of active metabolites may have

led to differences in several chronic studies (*e.g.*, Moskowitz and Smiley, 1982; Pecknold *et al.*, 1989), but this does not explain the reported advantages of buspirone against "cognitive" disruption after acute administration (*e.g.*, Seppälä *et al.*, 1982).

Unlike the effects of the 5-HT_{1A} receptor agonists on learning tasks, the effects of the longer-acting benzodiazepines, such as diazepam and CDZP, are well documented. Moreover, these benzodiazepines have been tested in experimental situations that involved both repeated acquisition and performance in pigeons (Thompson, 1975;), old world monkeys (Brocklehurst *et al.*, 1987) and humans (Bickel *et al.*, 1990; Desjardins *et al.*, 1982; Higgins *et al.*, 1987). The effects with diazepam in both monkeys and humans after oral administration were comparable to the effects found in this study for both i.m. and oral CDZP. In other words, there was little or no effect on either the overall rate of responding or percent errors until very high doses were tested and even then the effects were notably small when compared to oral administration of shorter-acting triazolobenzodiazepines such as alprazolam or triazolam. Brocklehurst *et al.* (1987), for example, found that patas monkeys responding under a multiple schedule of repeated acquisition and performance of conditional discriminations were more disrupted after triazolam administration than after diazepam administration; triazolam produced greater overall effects on rate and accuracy of responding than diazepam, and produced effects that were more selectively disruptive to acquisition. This finding in monkeys has also been reported in humans under similar behavioral conditions by Bickel *et al.* (1990). These investigators found that diazepam was less disruptive in terms of the magnitude of the effect on errors than either triazolam or alprazolam. The present results suggest that the effects of CDZP and alprazolam may show a comparable relationship in their ability to disrupt monkeys responding under a multiple schedule of repeated acquisition and performance.

As in our study, Brocklehurst *et al.* (1987) also found the longer-acting diazepam frequently produced comparable but small rate-deceasing effects in both components over a wide range of doses. Higgins *et al.* (1987) reported comparably small effects in both acquisition and performance after diazepam administration in 10 human subjects. This relatively uniform effect on response rate in both components may reflect the sedative properties of the 1,4-benzodiazepines (Randall *et al.*, 1960) and that is why decreases in response rate occur across both components. In our study, this small effect on rate with CDZP in both components was noted in three of five monkeys after i.m. administration and in four of five monkeys after oral administration. In only one case (monkey 76) did the highest dose for each monkey fail to produce a substantial rate-decreasing effect despite the fact that this monkey as well as the other monkeys were noted to have been sedated prior to the beginning of the session. More than likely, the somewhat greater effects found with oral CDZP on overall rate of responding are related to the higher blood levels produced by p.o. administration over i.m. administration. Greenblatt *et al.* (1974), in a direct comparison of i.m. and oral CDZP in human subjects, demonstrated that during the first 10 to 20 hr, blood concentrations were considerably higher after oral ingestion than after injection of the same dose. These authors concluded that i.m. CDZP is slowly absorbed, but after 72 hr, the completeness of absorption on the average was equivalent to that after the oral dosage.

The effects of CDZP in each monkey 24 hr after administration were strikingly similar regardless of the route of administration. Of particular note was the similarity in the effects on accuracy of responding even though the acquisition sequence for the 2 days was different. Although CDZP has a relatively long half-life (Greenblatt *et al.*, 1974; Randall, 1961), pharmacodynamic duration of action can be distinct from pharmacokinetic elimination half-life (Greenblatt, 1995). Additional studies that report the effects of this benzodiazepine on learning and performance procedures 24 hr after administration are needed.

Interestingly, alprazolam produced effects on response rate that were similar to CDZP, but its effects on the accuracy of responding were larger in magnitude and more selective across components than CDZP. As was found for CDZP, alprazolam comparably decreased response rate in both schedule components. Just as was found for CDZP, these decreases were relatively small even at the highest dose tested in three of five monkeys. However, this finding in monkeys is different from the effects reported by Bickel *et al.* (1990) in humans responding under a similar multiple schedule of repeated acquisition and performance. In their study, alprazolam produced greater rate-decreasing effects in performance than in acquisition. This difference in effect on response rate is surprising, given the similarity of the effects of alprazolam on percent errors in both humans and monkeys. Whether or not this difference in effect on response rate is due to differences in some behavioral variable (*e.g.*, second-order schedule, sequence length or timeout duration) or some other experimental variable such as route of administration (oral *vs.* i.m.) or species (monkey *vs.* human) remains to be determined. Friedman *et al.* (1991) have shown that there are differences in the pharmacokinetics of alprazolam in humans and African green monkeys when given orally. Although one would not expect these differences to necessarily account for differences in behavioral selectivity across schedule components, this remains a possibility. Clearly, one behavioral variable that does not appear to be contributing to this effect was the difference in the control rates of responding for each component. Similar to the human study by Bickel *et al.* (1990), data from three of five monkeys in our study showed that mean overall rates in performance were higher than mean overall rates in acquisition.

Although the repeated-acquisition base line in rats was as stable as the base line for monkeys, the effects obtained with buspirone and 8-OH-DPAT in rats were different from the effects obtained in monkeys in several ways. First, unlike the data obtained in monkeys, buspirone in rats was as disruptive, if not more disruptive, to the accuracy of responding than 8-OH-DPAT, regardless of the route of administration of 8-OH-DPAT. Moreover, although s.c. 8-OH-DPAT produced more graded effects and was more disruptive than i.p. 8-OH-DPAT to both rate and accuracy of responding, the magnitude of this effect at higher doses was not as great as that produced by i.p. buspirone. For example, at the highest dose of buspirone tested, all six rats had mean percent errors of more than 60%, whereas at the highest dose of 8-OH-DPAT tested, only two rats had mean percent errors of more than 60%. McCloskey *et al.* (1987) has reported a similar potency difference for buspirone when it was given s.c. and

i.p. to rats responding in a conflict procedure. In that study, s.c. administration also produced more potent effects on responding than i.p. administration. For buspirone, the difference in effect between the two routes of administration was taken as a further indication that a significant "first-pass" effect occurs with buspirone (Gammans *et al.*, 1982; McCloskey *et al.*, 1987). Whether or not the same is true for 8-OH-DPAT in both rats and monkeys remains to be determined.

A second difference that occurred for these two drugs between monkeys and rats was that both buspirone and 8-OH-DPAT produced rate-increasing effects in rats. Although these rate-increasing effects were most noticeable in subjects with low overall rates of control responding, there were instances where low s.c. doses of 8-OH-DPAT produced increases in rate in subjects with relatively high control levels of responding (see fig. 9). This finding is one of the few reported instances of rate-increasing effects for either buspirone or 8-OH-DPAT on schedule-controlled behavior maintained by food presentation under an FR schedule. Geller and Hartmann (1982) have reported rate-increasing effects after buspirone in rats responding under a variable-interval schedule of food presentation. In rats, specifically, buspirone has been reported to increase conflict responding (Geller and Hartmann, 1982; McCloskey *et al.*, 1987; Weissman *et al.*, 1984; Young *et al.*, 1987), but these rate-increasing effects have been found to be less than consistent or minimal on punished responding when compared to the benzodiazepines in monkeys and rats (see Gleeson and Barrett, 1990; McCloskey *et al.*, 1987; Witkin and Perez, 1989–1990).

The effects of CDZP in rats were also different from the effects obtained with CDZP in monkeys. Similar to buspirone and 8-OH-DPAT in rats, CDZP produced rate-increasing effects at certain low and intermediate doses, depending on the subject. Overall, CDZP produced rate-increasing effects at one or more doses in four of six rats. Such increases in response rate with CDZP have been reported in rats responding under several different operant schedules of reinforcement maintained by food presentation (Sanger and Blackman, 1976; Wedeking, 1968; Wedeking, 1974). However, responding under schedules of reinforcement that maintain lower overall response rates tend to be increased by CDZP more than responding maintained under schedules that maintain higher overall rates of responding such as an FR (*cf.* Sanger and Blackman, 1981). Sanger and Blackman (1976), for example, found that 2.5 and 5 mg/kg of CDZP increased rates of responding under variable-interval schedules but failed to increase responding under an FR-30 schedule. Wedeking (1974), in contrast, found that the same doses of CDZP increased VI rates but also increased FR response rates under a chained 10-sec differential reinforcement of low rates schedule (DRO), FR-25 schedule. In an earlier study, Wedeking (1968) had already demonstrated that CDZP increased response rates under a simple FR-25 schedule.

On the accuracy of responding in rats, CDZP produced dose-dependent error-increasing effects, but these effects generally occurred at the higher doses (18 and 32 mg/kg) that substantially decreased the overall rate of responding. In this regard, CDZP was similar to i.p. 8-OH-DPAT in rats. As indicated by the cumulative records in figure 11, both i.p. 8-OH-DPAT and i.p. CDZP tended to slow the rate of acquisition while producing some additional errors, but acquisition of the sequence clearly occurred during the session after administration of each drug. CDZP has been shown to disrupt both the acquisition and performance of a variety of complex behavioral procedures in rats [see Cole (1986) for a review]. In addition, Thompson (1973, 1975) has shown similar disruptions in accuracy with CDZP (20–40 mg/kg) in pigeons responding in a repeated-acquisition procedure involving a four-response sequence that was maintained by food presentation under a second-order FR schedule.

Unlike CDZP in monkeys, this drug in rats did not produce disruptive effects on either rate or accuracy 24 hr after drug administration. The reason for the shorter duration of effect in rats is probably due to the relatively short half-life of CDZP in rats compared with the half-life in humans or monkeys (Greenblatt *et al.*, 1974; Randall, 1961). In rats, the half-life of CDZP has been reported to be about 4 to 6 hr (Koechlin *et al.*, 1965; Randall, 1961) and occurs via a different metabolic pathway than has been determined for humans (Koechlin *et al.*, 1965).

In summary, the data obtained from our study indicated that all four drugs tested in monkeys dose-dependently disrupted response rate in both the acquisition (learning) and performance components of a multiple schedule. With buspirone and 8-OH-DPAT, however, the disruption of response rate generally occurred in acquisition before the disruption of response rate in performance, possibly indicating the relative absence of psychomotor disruptions produced by this class of drugs at these lower doses. In monkeys, for example, doses of 8-OH-DPAT as high as 2 mg/kg are required to induce a 5-HT_{1A} receptor-mediated motor disruption (Mizuta *et al.*, 1990). Regarding disruptions in accuracy of responding, each of the four drugs tested in monkeys disrupted learning to differing degrees (*i.e.*, 8-OH-DPAT > alprazolam > buspirone > CDZP). Differential effects on learning were most evident in the data for 8-OH-DPAT and alprazolam where large increases in percent errors occurred in the learning component, but little or no effect on percent errors occurred in the performance component. This substantial disruption to learning by a $5HT_{1A}$ agonist was also demonstrated by the fact that 8-OH-DPAT produced the largest increase in percent errors across all four drugs, and also produced larger differential disruptions of response rate.

Also of importance in these findings were the similarities and differences in the ability of these drugs to disrupt acquisition across species. In general, the effects obtained in monkeys most closely parallel those found in humans. With two of the three drugs that were tested in both monkeys and rats (*i.e.*, CDZP and 8-OH-DPAT), the overall effects in monkeys could not have been predicted from the data obtained with rats. For example, in rats, CDZP produced large error-increasing effects that did not occur in monkeys, and both CDZP and 8-OH-DPAT (s.c. and i.p.) produced rate-increasing effects that also did not occur in monkeys.

The differential effects produced by buspirone and 8-OH-DPAT on learning suggest an important role for drugs with $5HT_{1A}$ agonist properties in the study of learning. In fact, few drugs tested on a similarly complex behavioral base line have shown the selectivity for disrupting learning that 8-OH-DPAT demonstrated in this experiment. Although buspirone has 5-HT_{1A} agonist properties, it has also been shown to have dopaminergic antagonist properties, which might possibly explain the difference between buspirone's effect on learning compared to that of 8-OH-DPAT (*i.e.*, a smaller overall effect on learning

with buspirone). Additionally, our data from both species indicate that atypical anxiolytics with agonist properties at 5-HT_{1A} receptors are probably as disruptive to "cognitive" processes as the benzodiazepines. In other words, despite the absence of a psychomotor or sedative effect, putative terms such as "anxioselective" should not be taken to mean "least disruptive."

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